The Heat-Shock Response in *Serpula* Species at the Cellular and Molecular Level.

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I certify that this thesis is the true and accurate version of the thesis approved by the examiners.

Signed ....[redacted]..... Date 2.VII.99.

Director of Studies

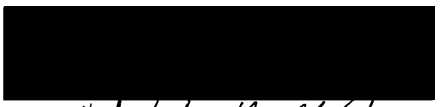
ABSTRACT

The study focuses on the effects of heat stress on *Serpula lacrymans* and *S. himantioides* (which have been previously shown to be relatively thermosensitive and thermotolerant respectively) in relation to the heat shock response. A part of this response is the production of heat-shock proteins (hsps). Comparative studies of chick embryo fibroblasts and *Saccharomyces cerevisiae* (wine strain, L-2226) were used to demonstrate possible differences between these three very distinct eukaryotic systems.

The aims of the project were to determine whether *S. lacrymans* and *S. himantioides* undergo the heat-shock response with respect primarily to induced thermotolerance and the production of hsps. The approach taken to establish the above was by systematic analysis of the different pathways involved in this response. Firstly, overall heat induced changes in protein and mRNA synthesis were analysed, in conjunction with the identification of specific proteins related to the heat-shock response, namely hsp 60, hsp70 which have been shown in other eukaryotic systems to play key roles in this response. Secondly, immunological studies were also used to detect changes in the pattern of protein ubiquitination, which resulted from heat stress. Thirdly, changes in hsp70 mRNA were monitored by RT-PCR. The cDNA and genomic amplimer products generated by RT-PCR and PCR respectively were cloned and sequenced to identify a putative of hsp70 gene sequence. Fourthly, the AMP-activated protein kinase (AMPK) cascade has been demonstrated to be activated in response to stress and steps were made to detect and identify AMPK activity in cellular extracts of both *Serpula* species. Finally, the effect of heat upon trehalose accumulation and mobilisation during the heat-shock response was studied as there is growing evidence to link increased thermotolerance to transient increase in trehalose levels, which is now considered to be a stress metabolite.

DECLARATION

I hereby declare that the work presented in this thesis was carried out by me at the University of Abertay, Dundee except where due acknowledgement is made, and has not been submitted by me for any other degree.

Signed 

Date 2nd July 1997

To my family,

for believing.

All for one and one for all,
United we stand,
Divided we fall.

From the Three Musketeers.

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Lastly, this piece of work is dedicated to my Dad, Mum, Gran and Granddad, which without their constant love and support throughout my life, I wouldn't be the person I am today. Granddad, I wish you were here to enjoy the moment.

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ABBREVIATIONS, SYMBOLS AND NOTATION

anhyd.	anhydrous
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
dH_2O	deionised water
DAB	diaminobenzidine
DEPC	diethyl pyrocarbonate
DTT	dithiothreitol
ddH_2O	double distilled water
2 x	double strength
g	gravity's
HRP	horseradish peroxidase
IEF	isoelectric focusing
pI	isoelectric point
kD	kilo Daltons
MEA	malt extract agar
EMM3	modified Edinburgh minimal media
M_r	molecular weight
N/C	nitrocellulose
NP40	nonidet P40
NRS	normal rabbit serum
1-D	one dimensional
OD	optical density
o/n	overnight
Rf	relative mobility

PMSF	phenylmethylsulfonylfluoride
PCIa	phenol: chloroform: isoamyl alcohol
PBS	phosphate buffered saline
PEG	polyethylglycol
RT-PCR	reverse transcriptase polymerase chain reaction
RT	room temperature
1 x	single strength
$Na_2H_2PO_4.H_2O$	di-sodium dihydrogen phosphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SBTI	soybean trypsin inhibitor
NaPPi	sodium pyrophosphate
NaF	sodium fluoride
SDS	sodium dodecyl sulphate (lauryl sulphate)
H_2SO_4	sulphuric acid
Temed	N, N, N', N'-tetramethylethylenediamine
TCA	trichloroacetic Acid
TBE	tris-boric acid-EDTA
TE	tris-EDTA
Train A, B	sublethal training regimes
Train C	optimum growth conditions
Tr	sublethal temperature training regime
Tr/HS	trained culture exposed to extreme temperature regime
Tr/HS/R	trained culture exposed to extreme temperature regime and placed into recovery at optimum growth conditions
2-D	two dimensional
u-p	ultra-pure

CHAPTER ONE

INTRODUCTION

1.0 The *Serpula* Species.

The two common species of *Serpula*, *S. lacrymans* (Schumacher ex Fr.) Gray and *S. himantioides* (Fr. ex Fr.) Karst, belong to a group of fungi classified as Basidiomycetes. The Basidiomycetes are a group of fungi within which are grouped most of the larger species: mushrooms, toadstools, puffballs, stinkhorns and bracket fungi (Coggins, 1980). Many of the important wood destroying fungi, are Basidiomycetes, which have large distinctive fruiting bodies. The wood destroying Basidiomycetes can be split into two groups depending on the type of enzymes secreted, brown- and white-rot fungi. The enzymes needed to breakdown cellulose and lignin, the two major components that make up wood, are cellulases and lignin degrading enzymes. *Serpula* species are brown-rot fungi and only secrete cellulases resulting in cellulose and similar compounds being broken down. The decay occurs as a darkening of the wood with the original structure broken up into cuboidal pieces of varying size (Coggins, 1980). This decay is known as brown rot. Decay of building timbers is commonly referred to as dry rot or wet rot. There is only one true dry rot fungus, *S. lacrymans*, the related species *S. himantioides* is mostly found in the wild (Bakshi and Choudhury, 1958; Bakshi et. al., 1958). The dry rot fungus *S. lacrymans* is a common cause of failure in building timbers, but at least two other major timber decay fungi, *Coniophora puteana* and *Fibroporia vaillantii* produce wet rot. These fungi develop where the timber becomes wetter than is usual in dry rot outbreaks (Coggins, 1980). Due to the invasive nature of the dry rot fungus, standard treatment involves the total replacement of infected wood often with chemically preserved stock.

Concerns have been raised in recent years regarding the safety of the many wood treatment systems, e.g. approval to use the widely used tri n-butyltin oxide has been withdrawn from the UK and there are concerns on the safety of using PCP derivatives (Carey, 1992). This has resulted in an increased awareness to develop new conceptual approaches to wood preservation and remedial treatment.

Efforts are being made to find new methods of treatment for the control and possible eradication of a range of wood decay fungi including *S. lacrymans*. The replacement of these toxic wood preservatives with more sophisticated methods is now becoming widely researched. New methods of detection and treatment, which are now being applied to detection and control of dry rot, include immunological detection (Glancy and Palfreyman, 1993; Toft, 1993; Burge et. al., 1994), biological control systems (Bruce and Highley, 1991; Palfreyman and Bruce, 1994; Score and Palfreyman, 1994a, 1994b) and heat treatment processes (Koch et. al., 1989; Koch, 1991). A rational approach to the design of remedial treatment will be greatly enhanced when in-depth analyses of the responses of molecular and physiological mechanisms to these remedial treatments are understood.

Falk (1912) undertook the first detailed scientific study of *S. lacrymans*. Until recently the main field of research has been based on physiological changes related to growth, morphology and substrate utilisation (Harmsen, 1960; Watkinson, 1971; Watkinson et. al., 1981; Bravery and Grant, 1985; Hegarty and Seehann, 1988; Venables and Watkinson, 1989; Schmidt and Moreth-Kebernik, 1989a, 1991a, 1991b; Paajanen and Ritschkoff, 1991). Molecular and immunological techniques are now being increasingly used to characterise the molecular mechanisms employed by this fungus (Palfreyman et. al., 1994, 1995). Studies have demonstrated that molecular differences can be detected between different strains of *S. lacrymans* (Schmidt and Moreth-Kebernik, 1989b; Palfreyman et. al., 1991; Vigrow et. al., 1991a, 1991b; Vigrow, 1992; Palfreyman and Vigrow, 1994). Although *S. lacrymans* and *S. himantioides* are regarded as morphologically similar they are nevertheless distinguishable on the basis of habitat and physiological activity. *S. lacrymans* differs from *S. himantioides* in habitat, primarily by *S. lacrymans* usually found in buildings and *S. himantioides* found growing in the wild. The two fungi also differ in the morphology of the sporophore and in culture, particularly in the texture and colour of the mat, with the formation of liquid drops prevalent on the mat of *S. himantioides* only (Bakshi and Choudhury, 1958).

Morphological differentiation of the *Serpula* species can be divided broadly into a number of morphologically and physiologically distinct forms of mycelium, associated with the vegetative and sexual states of growth. These two distinct states can be characterised by the formation of mycelium and strands associated with vegetative growth and fruit-body formation with sexual growth. Gregory (1984) defined the vegetative state "as an elaborate and wide variety of plectenchymatous structures which serve a purely vegetative function". Production of these structures occur in response to changing demands encountered by a dynamic mycelium growing under heterologous conditions, allowing different parts of a growing colony to fulfil separate and sometimes contradictory roles. The mycelium is capable of occupying complex niches within its ecosystem and is a subtle, changeable entity equipped with responses to varied and often contradictory environmental conditions. During the development of mycelium from a point source under initially homologous conditions, it is well documented that changes in patterns of morphogenesis occur leading to the establishment of a peripheral growth region containing undifferentiated leader hyphae, behind which are produced morphologically and functionally distinct mycelial regions which aggregate and adhere together forming strands (Butler, 1957, 1958). These strands give *S. lacrymans* the striking ability to spread over and within nutritionally inert substances, until a fresh nutrient resource becomes available (Jennings, 1982). Strands also have contradictory roles, even though primarily their role is a vegetative one. They can produce offshoots of vegetative mycelium which can switch to a mode of sexual development and form fruiting-bodies, but the strands themselves cannot directly produce these sexual structures (Nuss et. al., 1991). Both vegetative and reproductive states are influenced by the same abiotic environmental factors such as nutrition, temperature, light and aeration (Clark et. al. 1980; Hegarty and Seehann, 1987; Hegarty, 1991; Jennings, 1991).

1.1 Molecular Differentiation of *Serpula* Species in Relation to Stress Responses.

The purpose of this study is the investigation of the heat induced stress responses, in *Serpula* species and to relate these responses, if possible, to observable changes at the molecular and cellular levels. Differentiation and stress responses in prokaryotic and eukaryotic organisms are closely related and in some cases highly conserved (Hoffman and Parsons, 1991; Conway de Macario and Macario, 1994) A number of heat-shock proteins involved in this universally conserved stress responses are also conserved throughout evolution (Zeilstra-Ryalls et. al., 1992; Boorstein et. al., 1994). The occurrence of a stress response in these systems often leads to the initiation of similar forms of molecular differentiation. Stress is characterised by the response of a biological system to extreme environmental conditions. Once a stress is detected, organisms may attempt to evade it by a physiological response. If stress evasion does not occur at these levels, changes in metabolic processes may occur. The most rapid responses involve changes in enzyme activity, followed by changes in substrates and effectors. Slower metabolic changes involve enzyme synthesis or degradation processes, although these still occur in a few minutes. Temperatures influence the synthesis, stability and activity of enzymes, and also have substantial effects upon the equilibrium constants of biochemical reactions (Hochachka and Somero, 1984).

1.2 The Effects of Temperature Stress on Cellular Responses.

When eukaryotic or prokaryotic cells are exposed to elevated temperatures, they respond by synthesising a small number of highly conserved proteins known as the heat-shock proteins (hsps) or stress proteins. The heat-shock response is defined by the following criteria: A rapid, transient re-programming of cellular activities to ensure survival during periods of stress, to protect essential components against heat damage and to permit the resumption of normal cellular activities during the recovery period (Burton, 1986). The transient reprogramming of cellular activities can be correlated to the induction of hsps. In some organisms the production of hsps has been associated with an increase in thermotolerance, i.e., an enhanced ability of organisms to survive exposure to otherwise lethal temperatures (Plesset et. al., 1982; Plesofsky-Vig and Brambl, 1985; Carper et. al., 1987; Laszlo, 1988). Induced thermotolerance is defined as the process whereby exposure to mild heat stress, above the optimal temperature range, increases the thermotolerance of a wide range of organisms when subjected to a subsequent lethal heat treatment (Plesofsky-Vig and Brambl, 1985; Mackey and Derrick, 1986; Key et. al., 1993; Welte et. al., 1993). When cultured cells are given such pre-treatments, their resistance to killing by extreme heat increases dramatically (McAlister and Finkelstein, 1980). This increased resistance or induced thermotolerance has been observed in every organism in which it has been studied, ranging from bacteria to man (Schlesinger et. al., 1982; Craig, 1985).

Gerner and Schneider (1975) published the first study illustrating this phenomenon of induced thermotolerance and further studies to the present day are still attempting to define the complex systems involved in developing thermotolerance. A simplified model for this development has been proposed by Li and Hahn, (1980) whereby thermotolerance in mammalian cells can be divided into three processes: an initial triggering event; the expression of developing resistance and the gradual decay or disappearance of the resistance. In a simplified model, the various stages involved in

thermotolerance development can be correlated to changing heat kinetics. Studies involving the kinetics of heat killing and thermotolerance in a variety of mammalian cell lines have been illustrated in terms of an Arrhenius plot (Dewey et. al., 1977; Bauer and Henle, 1979). The three stages of the induced thermotolerance model were demonstrated as an Arrhenius plot by measuring mammalian cell survival (Li and Nussenzweig, 1996). Thermotolerance develops by triggering events that converts normal cells to the triggered state with rate constant k_1. This triggering event also probably involves the activation of the heat-shock transcription factor, HSF1, resulting in increased expression of hsps (Sorger, 1991; Abravaya et. al., 1992; Lis and Wu, 1993; Morimoto, 1993). The next stage of the process is seen as the triggered cells becoming converted into a thermotolerant state with a rate constant k_2. Temperatures approximately above 43 ^{0}C, k_2=0: the triggered cells remain sensitive to heat, and if transferred back to optimum conditions of 37 ^{0}C become converted to a thermotolerant state. This thermotolerant state is manifested by the elevated expression of hsps, enhanced protection and faster recovery from thermal damage. The final stage of the process occurs when the thermotolerant cells are placed back into optimal conditions. The cells slowly re-establish their sensitive state which is governed by rate constant k_3 (Li and Nussenzweig, 1996).

Another important aspect of the heat-shock response is the simultaneous shut down of most metabolic processes during the transitional period of stress (Schlesinger et. al., 1982; Plesofsky-Vig and Brambl, 1985), whereby the transcription of previously active genes and the translation of pre-existing messages are repressed. Heat-shock proteins are synthesised directly after organisms experience one of a wide range of environmental stressors and may be a particularly important component of the stress response (Hoffman and Parsons, 1991). DiDomenico et. al., (1982) demonstrated that as long as *Drosophila* cells were maintained at elevated temperatures, heat-shock proteins continue to be the primary products of protein synthesis. Once cells are returned to optimal

conditions, normal protein synthesis gradually resumes (DiDomenico et. al., 1982; Plesofsky-Vig and Brambl, 1985). This response has been shown to be universal among higher eukaryotic systems, suggesting that heat-shock proteins have a protective role against heat as well as other forms of environmental stress (reviewed in Lindquist, 1986; Lindquist and Craig, 1988; Morimoto et. al., 1990; Sanders, 1993).

It is becoming clear that many hsps are stress proteins, rather than specific responses to heat stress. These proteins can be induced by a variety of stress inducing agents, including amino acid analogues (Laszlo, 1988; Laszlo and Li, 1993), heavy metals (Sanchez et. al., 1992), hydrogen peroxide (Morgan et. al., 1986; Keyse and Tyrrell, 1987; Collinson and Dawes, 1992; Gropper and Rensing, 1993), arsenite (Caltabiano et. al., 1986) ethanol shock (Boon-Niermeijer et. al., 1988; Gropper and Rensing, 1993), glucose deprivation (Bataille et. al., 1991) and salt (Valera et. al., 1992; Volker et. al., 1992; Blomberg and Adler, 1993; Lewis et. al., 1995). The induction of stress proteins in relation to induced levels of tolerance has been shown not to be solely a part of heat stress, for example, proteins have been shown to correspond with heat shock but which overlap with proteins induced by salt shock (Harrington and Alm, 1988; Bhagwat and Apte, 1989). Mild treatments other than heat stress can also elicit increased resistance not only against higher doses of those particular stressors, but also confer thermotolerance. This was demonstrated in yeast cells exposed to ethanol (Plesset et. al., 1982) and metal oxides (Chang et. al., 1989).

This study focuses on the effects of heat stress on *S. lacrymans* and *S. himantioides* in relation to the heat-shock response. Organisms control their sensitivity to temperature and indeed other environmental stresses by the production of a heat-shock response. Within the basidiomycetes, the heat-shock response has been documented at the molecular level only in the plant pathogen *Ustilago maydis* (Holden et. al., 1989) and the wood-decay fungus, *Schizophyllum commune* (Higgins et. al., 1993), with several responses reported including the production of hsps, ubiquitination of proteins

and changes in the activities of intracellular proteases. A number of eukaryotic studies have indicated that a number of hsps may potentially play a role in the induction or the development of thermotolerance (Li and Laszlo, 1985; Solomon et. al., 1990; Lindquist and Kim, 1996). However, the precise function of individual hsps as well as the nature of the specific biochemical changes responsible for this phenomenon is still unclear (Jozwiak and Leyko, 1992). Angelidis et. al. (1991) showed direct evidence for the involvement of hsp70 in thermotolerance, with the production of clones of the CV1 cell line which had both enhanced capabilities for producing hsp70 and increased heat resistance. In addition, in transformed rat fibroblasts it was demonstrated that a constitutively expressed hsp70 gene dramatically increased thermotolerance The two-dimensional analysis of total cellular proteins from this study, also demonstrated that increased basal expression of hsp70 was the only substantial change in the protein profiles (Li et. al., 1991)..

The induction of the heat-shock response is relatively well understood, in comparison with its termination and the process of recovery from a heat-stressed state (Welch and Suhan, 1986). The restoration of normal patterns of protein synthesis in heat-shocked cells of *Drosophila* has been suggested to depend on a critical level of hsp70 (DiDomenico et. al., 1982), while Stone and Craig, (1990) present genetic evidence for the self-repression of 70 kD hsp in *Saccharomyces cerevisiae*, which also supports a role for hsp70 in the termination of the heat shock response. In *Escherichia coli*, the repression of hsp genes and resumption of normal protein synthesis depends on the presence of an intact homologue of hsp70, the dnaK protein (Tilly et. al., 1983).

1.3 Heat-Shock Proteins as Molecular Chaperones.

From the accumulating data it is evident that a number of hsps are directly involved in protein-protein interactions, such as protein folding, and have been subsequently termed 'molecular chaperones' (Ellis et. al., 1989). The general role of chaperones in protein folding is to bind to the

exposed interactive sites of the unfolded or nascent polypeptides and facilitate their folding or refolding (Horwich et. al., 1990). There are a number of chaperone families which include hsp60, 70 and 90.

The families of hsp60 proteins, found in all bacteria, mitochondria and plastids of eukaryotic cells, exhibit similar chaperone activity to hsp70, but are structurally distinct. They play a crucial role in catalysing the folding of unfolded proteins and assembling higher-order structures. The chaperonins belonging to the Hsp60 Family, a subgroup of chaperone proteins (Ellis and Van der Vies, 1991; Zeilstra-Ryalls et. al., 1991; Hartl, 1996) have large cylindrical complexes comprising of two stacked rings of seven to nine subunits each (Braig et. al., 1994; Hartl, 1994). They interact with early intermediates in the protein-folding pathway and mediate the acquisition of the native structure of newly synthesised proteins by releasing the substrate in an ATP-dependent process (Bochkareva et. al., 1988; Baneyx and Gatenby, 1992; Mayhew et. al., 1996; Roseman et. al., 1996). In periods of stress however, the levels of the hsp60/GroEL chaperone has been documented to increase dramatically in both *E. coli* and *S. cerevisiae* (Reading et. al., 1988; Cheng et. al., 1989). Martin et. al., (1992) demonstrated *in vitro*, protein denaturation at elevated temperatures in *N. crassa* was prevented by binding to hsp60, illustrating the possible role of hsp60 to stabilise pre-existing proteins during stress.

Hsp70s as molecular chaperones, are also involved in a number of different physiological functions which include the following: (i) The uncoating of clathrin-coated vesicles which are intermediates in the pathway of receptor mediated endocytosis, an hsp70 protein known as 'uncoating ATPase' disrupts clathrin-clathrin aggregates in an ATP-dependent reaction (Ungewickell, 1985; Chappell et. al., 1986; Rothman and Schmid, 1986); (ii) Mediators of protein translocation across membranes in mitochondria, chloroplasts and the endoplasmic reticulum (Kang et. al., 1990; Rassow et. al., 1994; Stuart, et. al., 1994; Cyr and Neupert, 1996); (iii) A component of the steroid hormone receptor

complexes (Silver et. al., 1993); (iv) Take part in the regulation of the prokaryotic sigma factor ^{32}s and eukaryotic heat-shock factor (Strauss et. al., 1990; Abravaya et. al., 1992; Liu et. al., 1993). Growing evidence points to hsp70 as having a central regulatory role of its own stress induced synthesis (Stone and Craig, 1990; Baler et. al., 1992). In cultured heat-shock treated mammalian cells it has also been proposed that increased levels of unfolded polypeptides, results in a reduction in free hsp70, leading to the induction of heat-shock gene transcription (Baler et. al, 1992; Beckman et. al., 1992; Li et. al., 1992).

The Hsp90 family differs from the other two families in that hsp90 regulates the function of folded proteins by binding to them by an ATP-dependent mechanism, which is possibly used by all three families (Schlesinger, 1990; Hartl, 1996). Hsp90 associates with numerous cytosolic and nuclear proteins involved in cell signalling, including steroid hormone receptors (Catelli et. al., 1985; Sanchez et. al., 1985; Picard et. al., 1990; Scherrer et. al., 1990). Hsp90 also interacts with hsp56 and hsp70, as components of hetero-oligomers or aporeceptor complex. This complex primarily consists of an hsp90 dimer and an hsp56 monomer (Rexin et. al., 1988; Rehberger et. al., 1992). Hsp70 appears to be transiently associated with aporeceptor-hsp90 complex during its assembly (Perdew and Whitelaw, 1991; Smith and Toft, 1993).

A number of these hsps play important roles during normal growth and development at optimum temperatures as molecular chaperones, but increased expression at higher temperatures allows growth and metabolism to continue. Examples, where the 'house keeping' functions of important hsps are 'commandeered' during periods of stress have been demonstrated for both hsp60 and 70 (Skowyra et. al., 1990; Buchner et. al., 1991; Gragerov et. al., 1992; Schroder et. al., 1993; Martin et. al., 1996). From the biochemical analysis of hsp function as a facilitator of protein folding it has been proposed that the common signal for hsp induction is protein denaturation and that one

possible function during periods of stress is to prevent or repair, denaturation damage (Craig and Gross, 1991, Sherman and Goldberg, 1996).

1.4 Heat-Shock Proteins and the Ubiquitin System.

Heat-shock proteins and products of the ubiquitin system, also appear to play important roles in the cellular response to abnormal or modified proteins. The ubiquitin system, a multicomponent, ATP-dependent pathway, which has been well characterised in reticulocytes and yeast, (Hershko and Ciechanover, 1992; Jentsch, 1992) is postulated to be responsible for the removal of abnormal proteins and for the rapid degradation of normal short-lived proteins. The degradation of these proteins is essential for modulating the levels of key enzymes and regulatory proteins within the cell. Proteins are committed to degradation by their ligation to ubiquitin, a highly conserved 76-amino acid polypeptide. Post-translational conjugation of ubiquitin to proteins has been suggested to be part of an ATP-dependent protein degradation pathway mediated by the 26S-protease complex (Hershko, 1988; Ciechanover, 1994). Finley et. al., (1984) demonstrated a link between ubiquitin and the heat shock response. Using the mouse cell cycle mutant, ts85, defective in conjugating proteins, they showed an accumulation of abnormal proteins at the non-permissive temperature of 39 ^{0}C. Under these conditions, they reported an induction of hsps, thus establishing at least a temporary association between the stress response and the ubiquitin system. Other studies have indicated that the rate of ubiquitin synthesis increases with heat stress (Bond and Schlesinger, 1985; Finley and Varshavsky, 1985). For example, in the case of heat-shocked chicken embryo fibroblasts, there is a 5-fold increase in ubiquitin mRNA synthesis (Bond and Schlesinger, 1985). Ubiquitin gene expression is inducible by heat-shock in *Xenopus laevis* embryos, with transient accumulation of ubiquitin mRNA with increasing temperature levels (Ovsenek and Heikkila, 1988). In yeast, ubiquitin is encoded by a multigene family comprising of UBI1, UBI2, UBI3, and UBI4. The UBI4

gene codes for a polyubiquitin precursor protein, which is rapidly induced by heat-shock and has been shown to be an essential component of the stress response system (Finley et. al., 1987). Levels of UBI4 transcripts increased with the synthesis of high levels of abnormal poypeptides (Mager and Ferreira, 1993).

Repair and prevention of protein denaturation due to stress also requires the presence of heat-shock proteins. Hsp 70 has the ability to bind to abnormal or modified proteins and possibly plays a role in the protection of other cellular proteins against heat-induced damage (Munro and Pelham, 1985). Regardless of the stress inflicted upon the cell, the accumulation of abnormally folded polypeptides within the cell leads to induced thermotolerance and the increased expression of hsps (Lee and Hahn, 1988). There is supporting evidence for this hypothesis: (i) Ananthan et. al., (1986), also demonstrated that by injecting abnormal proteins into *X. laevis* oocytes, an hsp70 hybrid gene was activated; (ii) Karlik et. al. (1984), also observed that in *Drosophila* cells expressing increased levels of a mutant actin, there was an increase in the level of hsps expressed. As high levels of abnormal or unfolded proteins accumulated within the cell, they become bound to hsp70, resulting in reduced levels of free hsp70 chaperones. As a result of this, the heat-shock transcription factor (HSF) was activated and the cell responded by increasing the expression of its heat-shock proteins (Baler et. al., 1992). During stress-free periods it has been proposed that the HSF is maintained in an inactive state via its interaction with a heat-shock protein. This has been further substantiated by Abravaya et. al., (1992) demonstrating, through *in vitro* studies, the functional interaction between the mammalian HSF and the cytosolic hsp70. In this hypothesis it is proposed that the primary trigger of a transient increase in hsp expression is the increase in the level of abnormal proteins present in a cell during periods of stress which results in the activation of the HSF.

1.5 Other Markers of Cellular Stress.

1.5.1 AMP and AMP-activated Protein Kinase Cascades

AMP-activated protein kinase (AMPK) is the central component of a protein kinase cascade that phosphorylates and inactivates key regulatory enzymes of several mammalian biosynthetic pathways (Corton et. al. 1994). It was discovered as a kinase, which inactivated HMG-CoA reductase, the enzyme which catalyses the key regulatory step in sterol/isoprenoid biosynthesis (Beg et. al. 1973). Elevation of cellular AMP levels in response to environmental stresses that cause ATP depletion, activates this kinase, both by direct allosteric activation and phosphorylation by an upstream kinase kinase, resulting in increased activation of greater than 100 fold (Hardie 1994). The biochemical characterisation of this kinase in rat liver indicates a single polypeptide chain of 63 kD which contains both the allosteric (AMP) and catalytic (ATP) sites (Carling et. al. 1987). It has been subsequently shown to phosphorylate and inactivate other metabolic enzymes involved in fatty acid and glycogen synthesis (Hardie and MacKintosh 1992). Evidence for the presence of this AMPK cascade system in higher plants has been demonstrated by detection and biochemical characterisation of a homologue of the mammalian AMP-activated protein kinase from cauliflower (MacKintosh et. al. 1992; Ball. et. al. 1994). In the yeast *S. cerevisiae*, the protein kinase *Snf1* encoded by the gene sucrose non-fermenting-1 (SNF1) and has been demonstrated to be closely related to the catalytic subunit of the mammalian AMPK (Corton et. al. 1994).

Environmental stresses including heat-shock, glucose starvation, anoxia and exposure to arsenite have all shown to cause ATP depletion (Chen et. al. 1986; Gasbarrini et. al. 1992; Corton et. al. 1994; Hardie et. al. 1994). Changes in the ratio AMP: ATP levels correlate with the activation of AMP-activated protein kinase via heat-shock and arsenite exposure (Corton et. al. 1994). This demonstrates a possible role of AMP-activated protein kinase in the cellular response to stress, by

effectively blocking non-essential biosynthetic pathways and conserving cellular ATP levels for essential processes such as ion pumping. Figure 1.1 illustrates the possible physiological role AMPK has upon biosynthetic pathways and subsequent ATP levels.

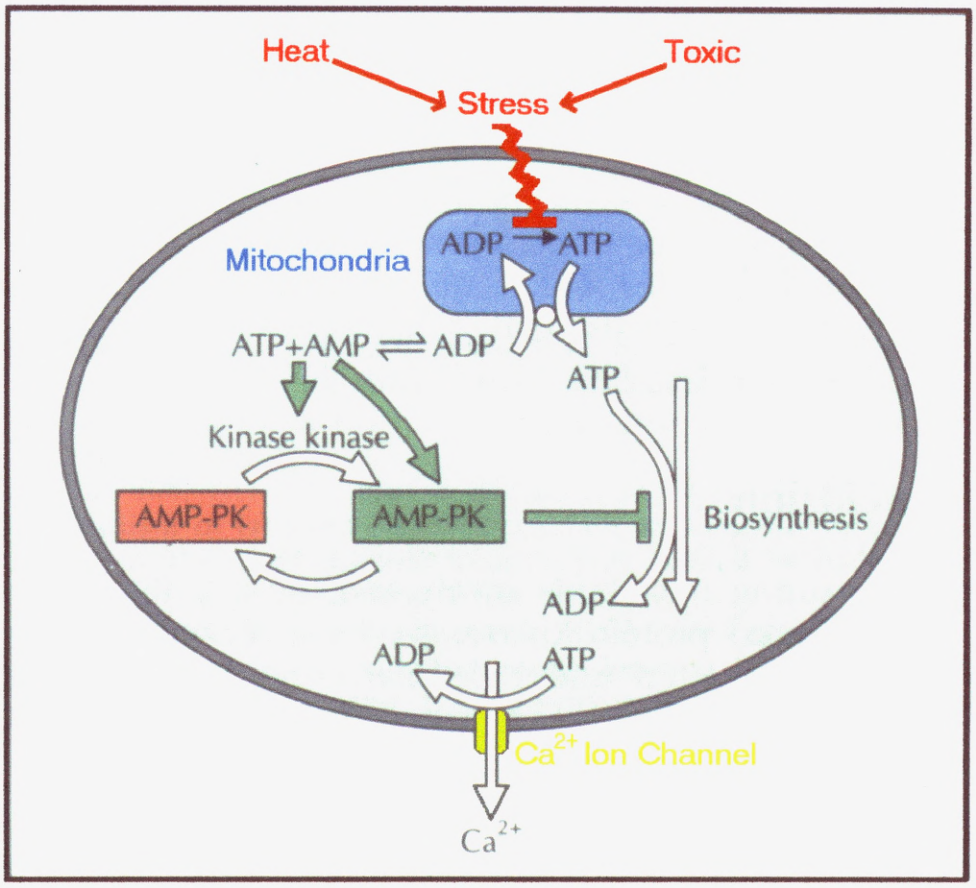


Figure 1.1. A model for the physiological role of AMP-activated protein kinase in response to environmental stress. Stress treatments, such as heat and arsenite shock, disrupt the ability of the mitochondria to phosphorylate ADP to ATP, through the adenine nucleotide translator. This disruption causes an increase in ADP:ATP ratio within the cytoplasm, resulting in an even larger rise in AMP:ATP ratio. Consequently, the rise in AMP activates the AMPK both by allosteric activation and by promoting the conversion of the inactive dephosphorylated (red) form to the more active phosphorylated (green) form, catalysed by the kinase kinase. This active form can phosphorylate in turn regulatory enzymes in a number of biosynthetic pathways, conserving ATP pools for more essential processes in the short term such as Ca^{2+} pumping. Adapted from Corton et. al. (1994).

1.5.2 Trehalase Activation and Trehalose Mobilisation During the Heat-Shock Response.

In *S. cerevisiae* the heat-shock response is characterised by rapid changes in cellular physiology and increased tolerance to stress conditions. Among the range of responses involved, two aspects are the induction of hsps and the rapid accumulation of the disaccharide trehalose which have been widely studied in this eukaryotic system. Trehalose is a non-reducing disaccharide of D-glucose, which is found in a number of eukaryotic systems including fungi such as *S. lacrymans* (Brownlee and Jennings, 1981). Although the biological function of trehalose is not fully understood, it is a storage carbohydrate found within the cytosol, and has also been recognised as a stress protectant in yeast (Keller et. al., 1982; Thevelein, 1984a; Van Laere, 1989; Wiemken, 1990). Rapid accumulation of trehalose can be induced by a number of environmental stresses similar to those which play a role in the induction of heat shock proteins, these include alcohol, hydrogen peroxide, and heat (Attfield et. al., 1987; Hottiger et. al., 1989; De Virgilio et. al., 1990). The biosynthesis and degradation of trehalose in yeast is a cyclic pathway in which it is synthesised by two enzymes, trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase and degraded by neutral trehalase (Figure 1.2). Accumulation or the mobilisation of trehalose in response to changes in temperature has been directly correlated with increases and decreases in thermotolerance in yeast cells, the rapid changes observed suggest that trehalose possibly acts as a thermoprotectant (Attfield et. al., 1987; Hottiger et. al., 1987a, 1989; De Virgilio et. al., 1990; 1991a).

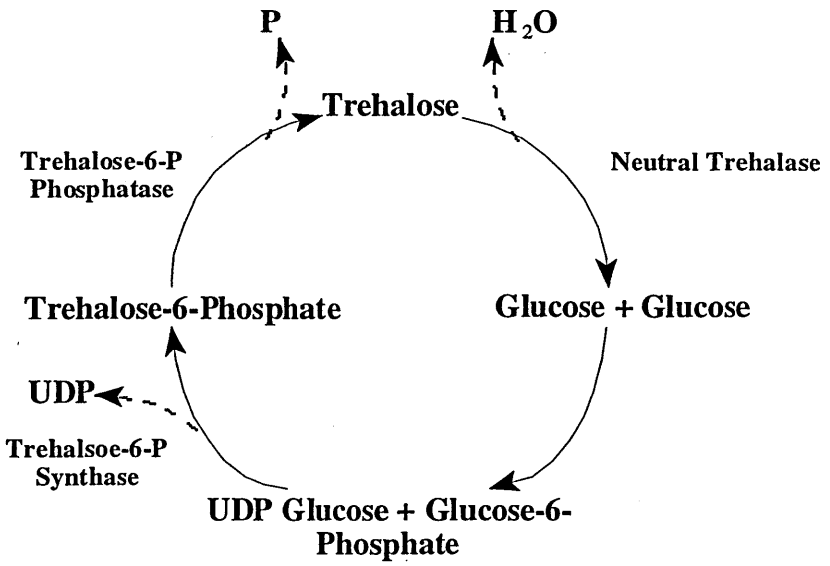


Figure 1.2. Pathway for the biosynthesis and mobilisation of trehalose.

Environmental stresses can affect fungi by triggering physiological and developmental mechanisms which are induced by changes at the post-translational and transcriptional level. In the case of trehalose, its mobilisation/degradation can be attributed to the regulatory enzyme, neutral trehalase. Trehalose mobilisation is a distinct feature of several developmental processes in fungi, such as spore germination and changes in fermentative growth, (Inoue and Shimoda, 1981; Uno et. al., 1983b Thevelein and Jones, 1983; Dewerchin and Van Laere, 1984; Thevelein, 1984b; Carrillo et. al., 1992; Soto et. al., 1995) in which there is a rapid change in neutral trehalase activity. Research has also shown trehalase to be involved in trehalose mobilisation in yeast cells under a variety of stressors (Van Laere, 1989; De Virgilio et. al., 1990). In yeast, there are two types of trehalase, a non-regulatory or acid trehalase that has a low pH optimum, high stability and does not regulate trehalose breakdown. The regulatory or neutral trehalase has a neutral pH optimum, lower stability and can be post-translationally activated by cAMP-dependent phosphorylation (Ortiz et. al., 1983a; Arguelles et. al., 1986; Thevelein, 1988). Evidence demonstrating the regulatory role of the RAS-adenylate cyclase signal pathway involved in trehalase activation comes from biochemical and

genetic studies where the addition of glucose to derepressed yeast cells causes a rapid increase in cAMP levels which triggers a cAMP-dependent protein phosphorylation cascade (Ortiz et. al., 1983b; Uno et. al., 1983; Thevelein, 1984; Thevelein and Beullens, 1985; Thevelein, 1991, 1992), leading to increased activation of trehalase.

In the fission yeast *Schizosaccharomyces pombe,* a similar cAMP-dependant pathway to the one present in *S. cerevisiae,* has also been implicated in the post-translational regulation of neutral trehalase (Carrillo et. al., 1992). Input of cAMP-mediated signal that triggers trehalase activation is induced by addition of glucose or other compounds to depressed cells, and the reversible protein phosphorylation is viewed as the mechanism responsible for transduction of such a signal into the active response (Carrillo et. al., 1994).

Stressing yeast cells by heat shock results in a rapid increase in enzyme activities which synthesise and degrade trehalose (trehalose-6-phosphate phosphatase/synthase and neutral trehalase respectively), suggesting increased turnover in trehalose (Hottiger et. al., 1987b; De Virgilio et. al., 1991b). There are a number of explanations to the rapid accumulation of trehalose in response to heat stress. Conflicting theories as to whether accumulation results either from heat-induced changes in the activity of these metabolic enzymes (Hottiger et. al., 1987b, De Virgilio et. al., 1991b) or possibly an increase in the concentration of the substrates for trehalose-6-phosphate synthase (Winkler et. al., 1991) have been put forward. Neves and Francois (1992), dispute the above findings with their own interpretation by which rapid accumulation of trehalose is induced by heat-shock, as a consequence of temperature dependent modifications in the kinetic properties of the enzymes, rather than changes in the amount of these enzymes. Evidence presently available concerning the accumulation of trehalose during a period of heat stress and its subsequent degradation during recovery, suggests that it plays a role in the stress response.

The relationship between changing trehalose levels and hsps has been substantiated by data suggesting that the 70 kD protein of the SSA subfamily of the hsp70 family, are negative regulators of heat-induced trehalose accumulation and have a possible role in recovery from a heat-stressed state (Hottiger et. al., 1992). The utilisation of trehalose as a biochemical marker to follow changes in heat stress in conjunction with the role of hsps in the recovery phase would give a greater understanding of the underlying mechanisms associated with this phenomenon.

1.6 Heat Stress and *Serpula* Species.

Most biological organisms can respond to a variety of external stimuli in specific ways involving biochemical changes, which in some instances mediate changes in differentiation. In the case of *S. lacrymans* heat stress caused by elevated temperatures has been investigated (Cartwright and Findley, 1934; Langvad and Goksoyr, 1965; Langvad, 1972; White et. al., 1995). The temperature sensitivity of *S. lacrymans* has been widely documented and temperatures of approximately 28 ^{0}C have been reported as being incompatible with growth (Jennings and Bravery, 1991); optimum growth occurs at 21 - 25 ^{0}C. Temperatures greater than 30 ^{0}C are lethal to *S. lacrymans* though different strains may respond to slightly different lethal temperatures. *S. himantioides* on the other hand has been shown to be thermotolerant in comparison, as it grows at 30 ^{0}C at a rate little lower than at 25 ^{0}C (Seehann and Riebesell, 1988)

It has been suggested that transient thermotolerance may be induced in *S. lacrymans* and *S. himantioides* by subjecting colonies to sublethal training temperature regimes (White et. al., 1995). In this study, fungal cultures were subjected to a variety of temperature regimes designed to investigate the induction of the heat-shock response and subsequent protection against exposure to extreme temperatures.

The possible mechanisms involved in the heat-shock response outlined in this chapter were investigated to ascertain whether or not they occur in *Serpula* species during induced transient thermotolerance and follow the classic heat-shock response. Comparative studies using two other eukaryotic systems as models, namely chick embryo fibroblasts and *S. cerevisiae* were included allowing any differences between the three systems to be evaluated. The following schematic (Figure 1.3) outlines the different pathways involved in the heat-shock response, and shows possible multi-regulatory roles associated with the response.

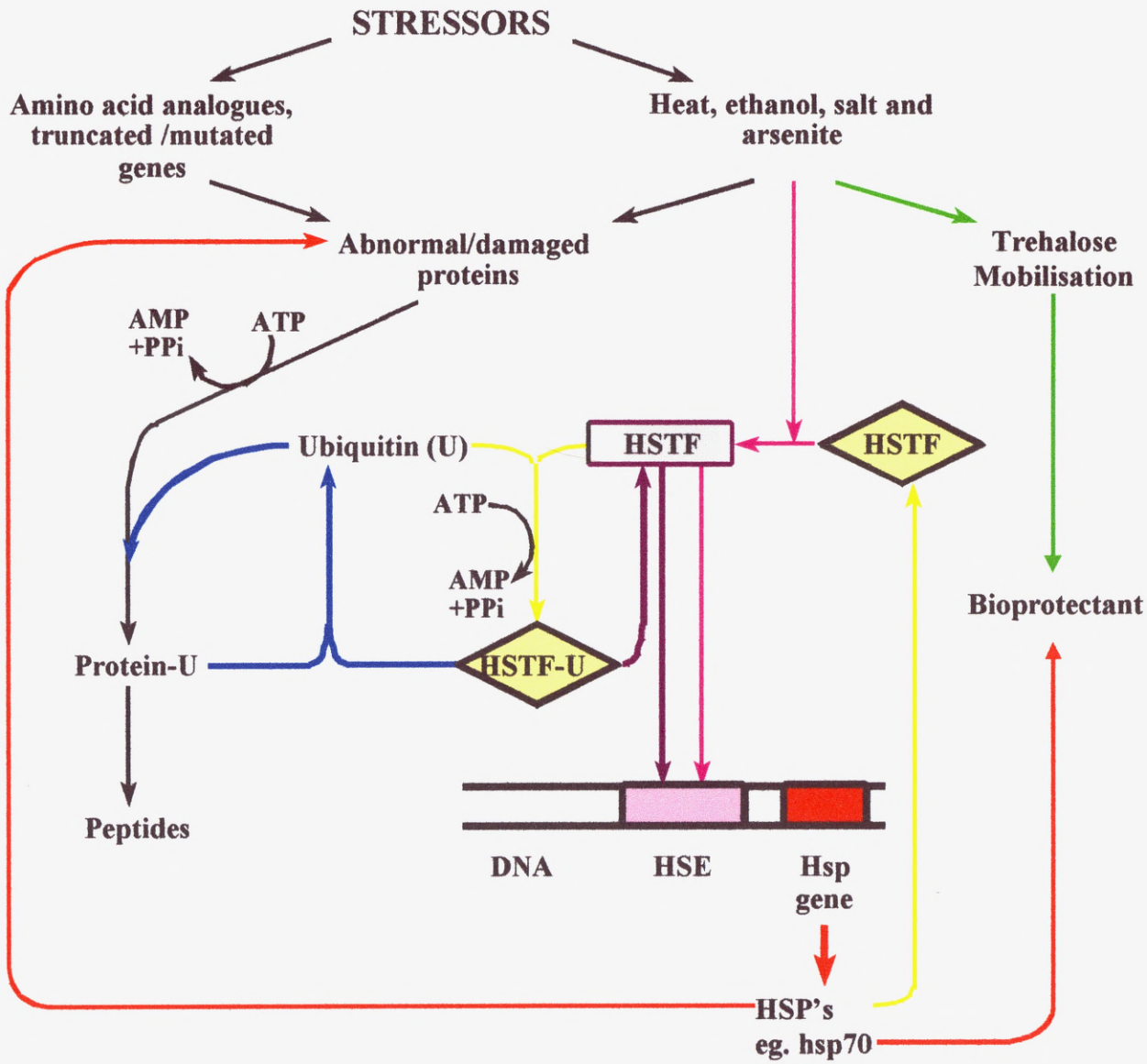


Figure 1.3. Possible pathways involved in the heat-shock response.

1.7 Aims and Objectives

The experimental rationale behind the research undertaken was to develop a system to explore the effects of sublethal temperature training regimes (White et. al., 1995) on cultures *S. lacrymans* and *S. himantioides* at the cellular and molecular level. The primary aim was to establish whether the sublethal temperature regimes used, effectively induced thermotolerance with respect to specific molecular changes involved in the development of the heat-shock response in the two *Serpula* species. This was achieved by investigating the various aspects of this response summarised in Figure 1.3.

The specific aims of the research were as follows: -

(i) Metabolic labelling studies utilising the radioactive isotope, ^{35}S-methionine were initiated to ascertain any overall changes in synthesised proteins. The labelling studies were to provide molecular evidence as to whether *S. lacrymans* and *S. himantioides* have the ability after pre-conditioning at sublethal temperatures, to survive an extreme heat treatment with the resumption of protein synthesis during the recovery period. Specific changes in the patterns of protein synthesis were investigated to provide possible evidence linking the induction of hsps to acquired thermotolerance, during the sublethal and extreme temperature regimes imposed.

(ii) To demonstrate any changes in mRNA transcripts expressed during the different temperature regimes by *in vitro* translation studies, with particular emphasis on the expression of possible hsp precursors.

(iii) The immunological identification of specific proteins related to the heat-shock response, in particular to detect cross reactive hsp60 and 70 antigens, which play key roles in this response.

(iv) To determine by immunological methods, whether changes in protein ubiquitination occur resulting from exposure to sublethal and extreme temperature regimes.

(v) The signalling pathway, the AMP-activated protein kinase (AMPK) cascade, has been shown to have a physiological role in conserving the ATP by inhibiting a number of biosynthetic pathways during periods of stress. Although this enzyme has been identified and characterised in rat liver extracts, and a homologue has been identified in higher plants and in the yeast *S. cerevisiae*, AMPK has not been investigated in any basidiomycetes to date. Work was carried out to establish whether this enzyme could be detected in a novel system such as the *Serpula* species.

(vi) Close examination of hsp 70 and its integral role in the heat-shock response has to date only been initiated in the basidiomycete *U. maydis* (Holden et. al. 1989) and *Agaricus bisporus* (Schaap et. al. 1996). Studies were undertaken to monitor specifically hsp 70 transcripts and the genomic DNA by RT-PCR and PCR respectively, with subsequent cloning and sequencing of the amplified products.

(vii) Studies were initiated to monitor the levels of trehalose, and its degradative enzyme trehalase, with respect to changes in temperature, and to also evaluate whether under the sublethal temperature regimes imposed, a direct correlation between increased levels in trehalose and increased thermotolerance could be ascertained.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Culture, Growth, Maintenance and Harvesting.

2.1.1 *S. lacrymans*, *S. himantioides* and *Coniophora puteana* Isolates Used in Studies.

The isolates of *Serpula* species used routinely in this study were *S. lacrymans* (Schumacher ex FR), Gray, FPRL 12C and *S. himantioides* (Fr. ex Fr.) Karst, Bb24. The isolate of *C. puteana* (Schumacher ex FR), Karsten, FPRL 11R was only used in the immunological studies.

2.1.1.1 Culture Maintenance.

S. lacrymans (12C), was routinely cultured at 22 ^{0}C in a cooled incubator (Gallenkemp cooled incubator), while *S. himantioides* (Bb24) and *C. puteana* (11R) were routinely cultured at 25 ^{0}C. The fungal isolates were obtained from the Building Research Establishment, Garston, U.K. and maintained on 2% (w/v) malt extract / 1.5% (w/v) agar. Media was autoclaved for 20 mins at 15 Psi. and cooled to 50 ^{0}C. The media was either poured into 9cm Sterilin Petri dishes to a depth of approximately 8 mm or used to prepare slopes in sterile universal jars for maintenance of long term stocks. Long term storage was in the form of slopes kept at 4^{0}C fridge (Lec), and sub-cultured every 6 months.

2.1.1.2 Culture Training Conditions Used for Analysis of Induced Thermotolerance and Extreme Heat Treatment.

The training regimes utilised in this study were first proposed by White et. al., (1995), in which it was suggested that transient thermotolerance may be induced in *S. lacrymans* (12C) and *S. himantioides* (Bb24) by subjecting colonies to sublethal temperature training regimes. Mycelial plugs, 5 mm in diameter were taken from the peripheral margin of 2 - 3 week old

stock plates and inoculated onto sterile 9 cm Petri dishes containing 20 ml of sterile low nutrient media (LNM) based on Hutterman and Volger, (1973) :- 2.5 g D-glucose (anhyd.); 0.5 g KH_2PO_4, 0.305 g $MgSO_4$-$7H_2O$; 0.25 g KCl, 0.004 g NH_4NO_3; 0.005 g $FeSO_4.7H_2O$; 0.004 g $Mn(CH_3COO_2).4H_2O$; 0.01 g $Zn(NO_3)_2.6H_2O$; 0.025 g $Ca(NO_3)_2.4H_2O$; 0.001 g $CuSO_4$ (anhyd.); all components dissoved in 500 ml of dH_2O.

Figure 2.1 A and B illustrates cultures of 12C and Bb24 respectively grown on minimal media at optimal conditions before sublethal training temperature regimes were imposed.

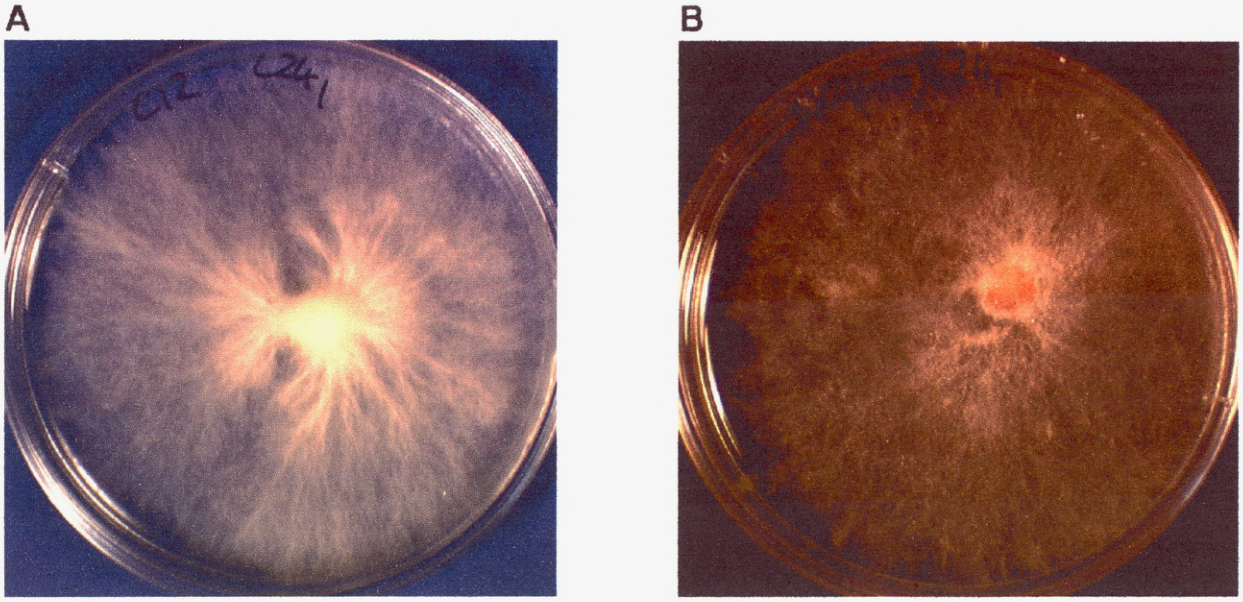


Figure 2.1 A and **B**. Liquid minimal media cultures of 12C (A) and Bb24 (B) grown at optimal conditions of 22 ^{0}C and 25 ^{0}C respectively prior to exposure to sublethal training regimes.

Liquid cultures were placed in tin boxes and incubated at the appropriate optimum temperatures for *S. lacrymans and S. himantioides* until the mycelium had reached approximately 80 mm in diameter. At this point the cultures were placed at the varying temperature regimes outlined in Figure 2.2.

Heat treatment of *S. lacrymans* (12C): -

For *S. lacrymans* (12C), Train A and B correspond to cultures placed at sublethal training regimes at 28 ^{0}C for 24 hrs and 25 ^{0}C for 48 hrs respectively prior to extreme heat treatment at 40 ^{0}C for 2 hrs. Train C relates to cultures grown at optimum conditions (22 ^{0}C for 24 hrs) again prior to heat-shock treatment. After extreme heat treatment all the cultures were returned to optimum conditions, 22 ^{0}C for 24 hrs to recover.

Heat treatment of *S. himantioides* (Bb24): -

For *S. himantioides* (Bb24), Train A and B correspond to cultures placed at sublethal temperatures of 28 ^{0}C and 30 ^{0}C, respectively for 48 hrs prior to extreme heat treatment at 40 ^{0}C for 3 hrs. Train C again responds to optimum conditions (25 ^{0}C for 24 hrs), prior to extreme heat treatment. After extreme treatment all the cultures were placed back into optimum condition for a period of 24 hrs to recover.

Heat Treatment of *C. puteana* (11R): -

Cultures of *C. puteana* were incubated under the exact conditions for Bb24 control cultures exposed to Train C temperature regimes.

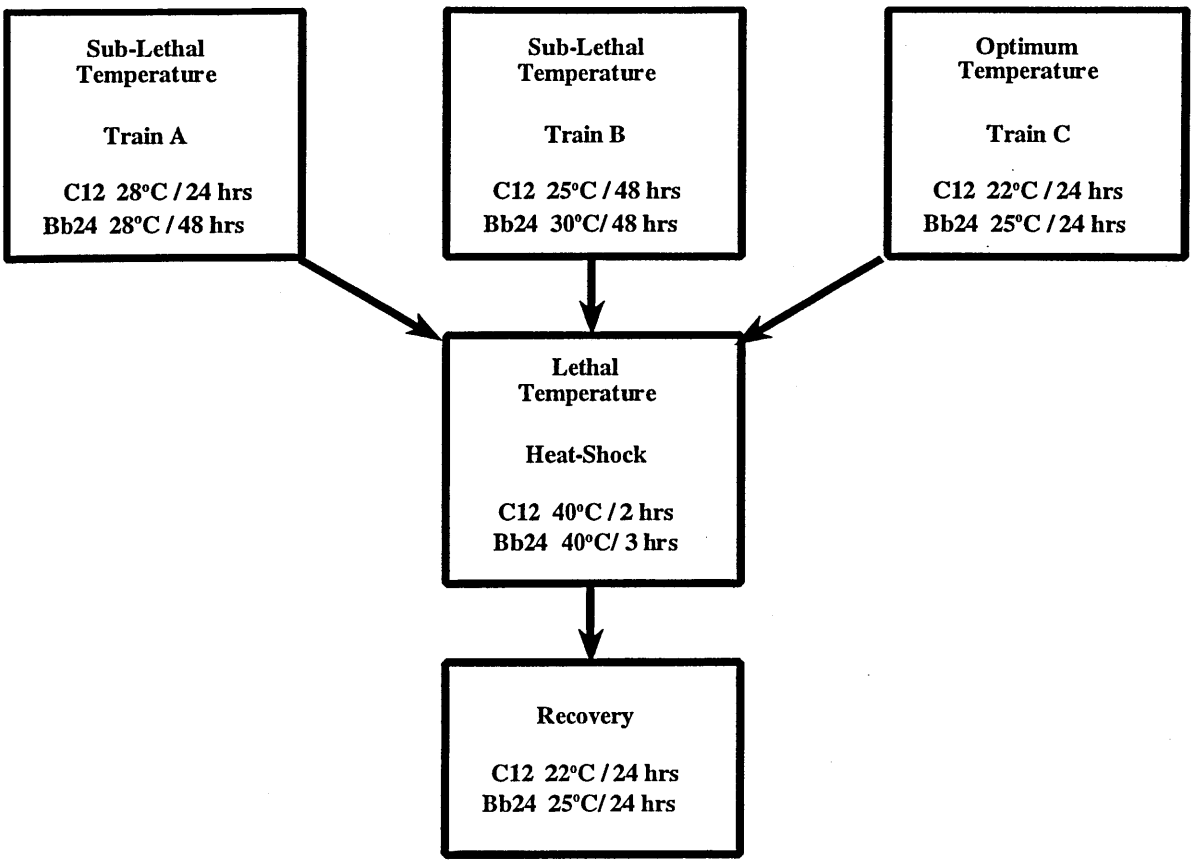


Figure 2.2. Flow diagram representing the experimental outline for the exposure of *S. lacrymans* (12C) and *S. himantioides* (Bb24) cultures to the varying training regimes.

2.1.1.3 Metabolic Labelling of Cultures

To monitor protein synthesis, Bb24 and 12C cultures were labelled with L-[^{35}S]-methionine (74 MBq stock of L-[^{35}S]-methionine (cat. no. SJ204, Amersham International Limited, UK), was added at a final concentration of 8.2 MBq per 20 ml of growth medium for both Bb24 and 12C cultures) for 3 hrs, immediately prior to harvesting, with the exception of 12C extreme heat-treated cultures which were labelled for only 2 hrs.

2.1.1.4 Harvesting of Cultures

LNM grown cultures were harvested during the training, extreme treatment and recovery periods as follows: the inoculation cores were discarded and mycelial mats, briefly blotted dry on Whatman 3MM paper to remove excess liquid and quickly immersed in liquid nitrogen (in the case of labelled material, the immersion into liquid N_2 stopped any further incorporation of the radioisotope). Frozen mycelium was stored at -20 ^{0}C or under liquid nitrogen (where stated) until further processing was required. The non-labelled 11R cultures were harvested in the same manner as Bb24 and 12C.

2.1.2 *Saccharomyces cerevisiae.*

2.1.2.1 Culture and Maintenance.

The wine strain, L-2226 was used in this study as a fungal control for comparing the production of hsps under heat stress. Routine maintenance on media slopes 3 % (w/v) MEA, 0.5 % (w/v) mycological peptone and 1.5 % (w/v) agar at 30 ^{0}C. Liquid stocks were prepared by inoculating 50 ml flasks containing EMM3 media. with a sterile loop with yeast cells. The liquid stocks were maintained at 30 ^{0}C overnight until the cells had reached a late exponential growth phase. The cell count was measured using a coulter counter (Coulter Electronics Ltd.) according to the manufacturer's instructions, and was approximately 20 x 10^6 cells/ml of medium. 3 ml of the stock was inoculated into 50 ml of fresh EMM3 medium and incubated for approximately 16 hrs prior to undergoing the training regimes. For comparative work it was essential that the yeast stocks underwent similar training regimes to that of *Serpula* species.

The components for Modified Edinburgh Minimal Medium (EMM3) were prepared separately and mixed aseptically just prior to use.

Base Medium

Glucose	20 g
Ammonium Sulphate	5 g
$NaHPO_4$	1.5 g
dH_2O	500 ml

Solution was pH to 5.5 and autoclaved for 20 min at 15 Pi.

Salt Medium

KCl	0.5 g
$MgSO_4.7H_2O$	0.5 g
$CaCl_2.6H_2O$	0.015 g
dH_2O	500 ml

Solution was pH to 5.5 and autoclaved for 20 min at 15 Pi.

The base and salt medium were mixed aseptically to give 1 l of medium, to which 1 ml of filter sterilised 1000 x stocks of trace elements and vitamins were added at a final concentration as follows:-

Trace Elements

H_3BO_3	0.5 mg
$MnSO_4.H_2O$	0.4 mg
$ZnSO_4.7H_2O$	0.4 mg
$FeCl_3.6H_2O$	0.2 mg

$H_2MoO_4.H_2O$	0.16 mg
KI	0.16 mg
$CuSO_4.5H_2O$	0.04 mg
Citric Acid	1 mg

Vitamins

Inosital	10 mg
Nicotinic Acid	10 mg
Calcium Pantothenate	1 mg
Biotin	0.1 mg
Pyridoxine-HCl	0.4 mg
Thiamine-HCl	0.4 mg
p-Aminobenzoic Acid	0.2 mg

2.1.2.2 Heat Treatment Regimes.

Duplicate flasks of exponentially growing yeast cells underwent the following heat treatment regimes by placing them in pre-heated water baths at the appropriate temperatures for 1 hr as illustrated in Figure 2.3.

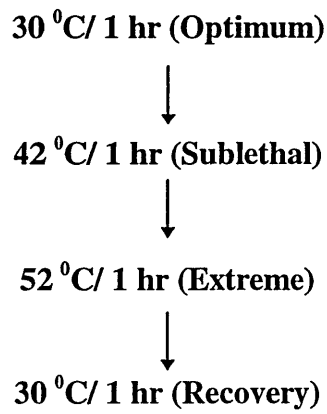


Figure 2.3. Heat treatment regimes employed in yeast studies. The optimal regime for actively growing yeast cells is between 23 - 36 ^{0}C (McAlister and Finkelstein, 1980). The temperature of 42 ^{0}C has been used in other studies to demonstrate induced thermotolerance in yeast cells (Birch, 1997). While exposure of control cultures to 52 ^{0}C has been demonstrated to be an extreme temperature which can effectively kill (McAlister and Finkelstein, 1980, Hottiger et. al., 1994a).

2.1.2.3 Cell Harvest.

Yeast cells were harvested by filtering through 0.2μm Whatman Anodisc 25 membrane filter and washed 2 x ice cold dH_2O. The filter containing yeast cells were transferred into centrifuge tubes and immersed in liquid nitrogen until cells were snap-frozen. The frozen centrifuge tubes were stored at -20 ^{0}C until further processing.

2.1.3 Chick Embryo Fibroblasts (CEF).

2.1.3.1 Culture and Maintenance of CEF Cell Line.

The chick embryo cell line was established from 9-12 day old embryos and cultured onto supplemented Dulbecco's Modified Eagles Medium (DMEM): 100 ml DMEM (Sigma); 10 ml Foetal calf serum; 3.1 ml (100 X stock)1 X Penicillin/Streptomycin; 200 ml L-glutamine. The cell line was maintained at 37 ^{0}C water saturated atmosphere containing 5 % CO_2 incubator. Once the cell monolayer was confluent, the cells were split and subcultured in fresh medium onto 6 cm^2 sterile Petri dishes at appropriate dilutions.

2.1.3.2 Heat Treatment Regimes.

The CEF cells were heat-shocked by directly placing them into a 42 ^{0}C incubator (saturated atmosphere containing 5 % CO_2) for 1 hr and placed back into optimum conditions of 37 ^{0}C to recover for 1 hr. Cultures were harvested for biochemical analysis after each the heat treatments imposed.

2.2 Protein Separation by 1-Dimensional Electrophoresis.

2.2.1 Sample Preparation of Mycelium.

All steps were performed on ice at 4 ^{0}C.

2.2.1.1 Sample Preparation - Method 1.

Frozen samples of mycelium were initially ground to a fine slurry in 200 µl of Homogenising buffer A, ice cold Phosphate Buffered Saline (PBS 10mM, pH 7.4, pre-made tablets from Sigma containing 1 mM PMSF, 1 mM benzamadine, 1 mM EDTA) using a pestle and mortar on ice at 4 ^{0}C. The homogenate was microcentrifuged (Centurion 8000 Series, Norlab Instruments Ltd.) for 10 min at 12, 500 rpm at 4 ^{0}C and stored at -20 ^{0}C.

2.2.1.2 Sample Preparation - Method 2.

Homogenisation of Frozen Mycelium Using Seesand.

Pooled colonies of upto three replicates of frozen mycelium were homogenised in an eppendorf with a micro pestle and 1\10th volume of acid washed Seesand (Fluka) and 300 µl of homogenising buffer B (50 mM Tris-HCl, pH7.4 containing the following :-10 % (v/v) Glycerol, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM NaPPi, 0.02% (w/v) Brij, 1 µg/ml SBTI (dissolved in u-p dH_2O), 1 mM DTT, 0.1mM PMSF, 1 mM benzamadine) until the mycelium had been thoroughly broken up (approximately 30 sec). The sample was microcentrifuged for 5 min at 10, 000 *g* at 4 ^{0}C. The resultant supernatant was divided into aliquots, snap frozen in liquid nitrogen and stored at -20 ^{0}C until further required. For preparation of 2D SDS-PAGE samples, the homogenising buffer was substituted for 2-D

sample buffer (2.5 ml 0.5M Tris, pH 6.8, 2.5 ml 10 % (w/v) SDS, 2.5 ml 1 M DTT, 17.5 ml u-p dH_2O).

Further refinement of the procedure by concentrating the amount of protein present in a given sample was achieved by freeze drying of the supernatant. 100µl of supernatant was added to an Eppendorf. Samples were freeze dried (Edwards Freeze Dryer Micro Modalyo) for 1 hr, after which the precipitated material was stored at -20^{0}C until further processing.

2.2.1.3 Preparation of Radioactive Material.

Snap frozen labelled mycelia were disrupted in 300 µl of ice cold homogenising buffer B using Seesand on ice at 4 ^{0}C. Extracts were centrifuged (IEF Centra-4R) for 10 mins at 3000 *g* set for 4 ^{0}C, the resultant supernatants refrozen in liquid N_2 and stored at -20 ^{0}C until further processing. For long term storage, supernatant aliquots were stored under liquid nitrogen.

2.2.1.4 Radioactive Incorporation of ^{35}-S-methionine - TCA Precipitate and Scintillation Counting.

The radioactivity incorporated into proteins was determined by 10 % (w/v) TCA precipitation of labelled cell extracts, followed by the counting of radioactivity in sample precipitates by means of a LKB Wallace 1216 Rackbeta scintillation counter. 5 µl of labelled sample pipetted onto Whatman glass fibre disc (GF/C) and air dried. The filters were plunged into ice cold 10 % TCA solution and left for 30 mins after which the discs were washed in fresh 10 % cold TCA solution 3 x 5 mins. Excess liquid was drained off onto blue roll and the discs were transferred into clean 10 ml beakers and rinsed with ice cold absolute ethanol 3 x 5 mins. The washed discs were air dried as before and transferred into scintillation vials containing 4 ml of Ecosynt A (National Diagnostics). The amount of radioactive counts present was measured by

a scintillation counter (LKB Wallace, 1216 Rackbeta Liquid Scintillation Counter linked to a Wilker Digital Decwriter IV) which was programmed to read counts for [^{35}S]L-methionine.

2.2.1.5 Determination of the Protein Content Present in the Fungal Culture Supernatants.

The amount of protein present in non-labelled samples was determined by the Pierce protein micro-assay (Pierce Coomassie Protein Assay Reagent) based on the Bradford method (Bradford, 1976) according to the manufacturers instructions

2.2.2 Sample Preparation of Yeast Cells for Analysis.

2.2.2.1 Sample Preparation of Non-labelled Material.

Membrane filters containing yeast cells were thawed and resuspended in 300 µl of homogenising buffer C and vortex mixed for 2 x 30 sec (PBS containing a cocktail of protease inhibitors:-1 mM benzamadine, 1 mM EDTA, 1 mM PMSF, 10 mM NaF, 10 µg/ml pepstatin A). The cells were transferred into Eppendorf tubes containing 0.75 g of pre-cooled sterile glass ballotini beads (size 0.49-0.70 mm, Jencons Scientific Ltd.) and vortex mixed for 5 x 1 min intervals. The cell lysates were centrifuged, collected and stored at -20^{0}C until further processing. The proteins were concentrated by freeze drying as described in Section 2.2.1.2.

2.2.2.2 Determination of the Protein Content of Yeast Cells.

The amount of protein present in samples were determined by the Pierce protein micro-assay based on the Bradford method (Bradford, 1976) as described in Section 2.2.1.5.

2.2.3 Sample Preparation of Chick Embryo Fibroblasts for Analysis.

The growth medium was removed and the cell monolayer was rinsed 2 x sterile PBS. The petri dishes were inverted onto paper roll to allow the excess liquid to drain off. After which 500 µl of 2-D sample buffer was added to the monolayer scraped with a sterile blue tip with the end cut off. The sample was transferred to a fresh eppendorf and microcentrifuged 10, 000 *g* for 3 mins and stored at -20 ^{0}C until required. Protein content was measured as described in Section 2.2.1.5.

2.2.4 Electrophoretic Separation of Proteins by 1-D SDS-PAGE.

The equipment used for the mini-electrophoresis system were as follows:- FEC 185 Dual Mini-Vertical Gel System, (Fisons Plc.) Mighty Small System (Hoefer Instruments Ltd.), Mini Protean II System (Bio-Rad) and a microcomputer electrophoresis powerpack unit (Consort E445). Plates, spacers and combs were soaked in concentrated Decon 90 overnight, scrubbed gently with a soft toothbrush to remove any acrylamide particles from the surface, rinsed thoroughly in hot running water, soaked briefly in 0.1 % H_2SO_4 and finally immersed in u-p dH_2O. The glass plates were then dried with 70 % absolute alcohol and assembled together according to the manufacture instructions. Acrylamide/Bisacrylamide solutions supplied by Fisons. All solutions were filtered through Whatman No. 1 prior to use and stored in the dark at 4 ^{0}C. Phigel 3 (37.5:1 acrylamide: N, N-methylenebisacrylamide) 40 % w/v stock solution.

2.2.4.1 Preparation of Gels.

The proteins extracts were analysed by 1-D SDS-PAGE based on the method of Laemmli (1970). The single concentration gels (37.5: 1 acrylamide: bis-acrylamide) used consisted of 12.5 % resolving gel with a 5 % stacker.

Resolving Gel Buffer

45.38 g Tris and 1.00 g SDS dissolved in dH_2O, pH adjusted to 8.9 with concentrated (conc.) HCl before being made up to a final volume of 250 ml. Stock solution stored at 4 ^{0}C, aliquots at -20 ^{0}C.

Stacking Gel Buffer

14.73 g Tris and 1.00 g SDS dissolved in dH_2O, pH adjusted to 6.7 with conc. HCl, before being made up to a final volume of 250 ml. Stock solution stored at 4 ^{0}C, aliquots at -20 ^{0}C.

12.5 % Resolving Gel Mix

Acrylamide	7.5ml
Resolving gel buffer	6 ml
dH_2O	8 ml

12 ml of the stock gave enough to pour two mini-gels, polymerisation was activated by the addition of the following:-

10 % (w/v) ammonium persulphate	100 µl
TEMED	5 µl

The resolving gel mix was poured to 2 cm from the top of the glass plates. Water saturated butanol was overlaid onto the freshly poured resolving gel mix to prevent desiccation during

polymerisation. Once set gel is set the surface was rinse with 1:4 dilution of the resolving gel buffer to remove any residue of the butanol. If the gels were not used immediately, they were overlaid with 1:4 resolving gel buffer and stored overnight at 4 ^{0}C until required. When samples were ready to be loaded, the 1:4 resolving gel buffer was removed from the top of the gel, the 5 % stacker was poured to the top of the glass plate rim and the 10 well comb pushed down into place. Once the gel had set, the samples were loaded into the wells.

5 % Stacking Gel Mix

Acrylamide	(40 % solution)	3 ml
Stacking gel buffer		6 ml
dH_2O		14 ml

6 ml of the stock gave enough to pour two stackers and the polymerisation was activated by the following:-

10 % (w/v) ammonium persulphate	50 µl
TEMED	2.5 µl

2.2.4.2 Sample Preparation and Loading.

The supernatant was prepared for electrophoresis by 1:1 ratio with 2 x SDS sample buffer (0.31 ml 20 % (w/v) SDS, 0.24 ml 1 M Tris, pH 6.8, 0.3 ml 1 M DTT, 0.3 ml, Glycerol, 50 µl 0.2 % (w/v) Bromophenol blue, dH_2O0.345 ml, Laemmli, 1970) to give a 1x strength containing the following concentrations:- 2 % (w/v) SDS; 0.1 M DTT; 0.08 M Tris, pH 6.8; 10% (v/v) glycerol) to one part supernatant. The samples were boiled for 3 mins and microcentrifuged for 5 mins at 12, 500 rpm, and were subsequently loaded as follows: - radiolabelled and non-labelled samples were normalised for total protein content.

Standard proteins (Dalton SDS-7, Sigma, molecular weight ranges 14.2 - 66 kD, Sigma), low (^{14}C) Methylated Rainbow Markers (Amersham International, molecular weight ranges 2.35 - 46 kD) and high (^{14}C) Methylated Rainbow Markers (Amersham International, molecular weight ranges 14.3 - 220 kD). Standards were prepared according to the manufacture's instructions and stored at -20 ^{0}C in 20 µl aliquots. Prior to use the SDS-7 markers were diluted 1:10 with 2 x cracking buffer and heated at 100 ^{0}C for 3 mins, the low and high molecular weight markers were diluted 1:3 with 2 x cracking buffer and treated as for the standard protein markers. The prepared markers were microcentrifuged for 3 mins at 10, 000 *g*, just prior to loading.

2.2.4.3 Protein Separation.

The electrode tank buffer was made up to 5 x strength (15.15 g Tris, 72 g Glycine, 5 g SDS, 1 l dH_2O) and diluted to 1 x with u-p dH_2O before a gel run. The stock was stored at 4 ^{0}C. Proteins were separated on the single concentration 1-D polyacrylamide slab gels at a constant current of 15 mA per gel for approximately 2 hrs at room temperature with cold running water used as a cooling system. Following electrophoresis, the gels were either stained (Section 2.4) or electrophoretically transferred (Section 2.5) to nitro-cellulose membrane for further analysis.

2.3 Protein Separation by 2-Dimensional Gel Electrophoresis.

The protocols used for analysis of proteins by 2-D electrophoresis were based on the methods originally set out by O'Farrell (1975).

2.3.1 Preparation of Samples and 2-D Markers for First Dimension Tube Gel Electrophoresis.

2.3.1.1 Sample Preparation - Method 1.

Extraction of labelled and unlabelled intracellular proteins from harvested cultures for 2-D electrophoresis was prepared in the first instance as for the routine sample preparation for 1-D analysis (Section 2.2.1). Concentration of proteins in samples was achieved by freeze drying of cell lysates (Section 2.2.1.2) and the resultant lyophilised material was reconstituted in 10 - 30 µl of 2-D sample buffer (2.5 ml 0.5M Tris, pH 6.8, 2.5 ml 10 % (w/v) SDS, 2.5 ml 1 M DTT, 17.5 ml u-p dH_2O) and stored at -20 ^{0}C until required.

2.3.1.2 Sample Preparation - Method 2.

The method of extraction was based on homogenising frozen mycelium with Seesand (Section 2.2.1.2). The resulting supernatant from this extraction was freeze-dried and reconstituted at RT in diluting buffer (9.5 M u-p Urea, 5 % v/v NP40, 2 % v/v Ampholines pH 3.5-9.5, 50 mM DTT, mixture was filtered through a 0.45 µm Whatman disc and stored as frozen aliquots). Samples were either run immediately or stored at -20 ^{0}C.

2.3.1.3 Preparation of 2-D Marker Proteins.

Commercially pre-mixed 2-D markers from Biorad (cat. 161-0320) were prepared and used according to the manufacture instructions.

2.3.2 Separation of Proteins by Non-Equilibrium pH Gradient Electrophoresis (NEPHGE) in the First Dimension.

Samples were separated in the first dimension by non-equilibrium pH gradient electrophoresis (NEpHGE) as essentially described by O'Farrell (1975). All buffers and overlay solutions were filtered through a 0.45 μm Whatman disc and stored as frozen aliquots at -20 ^{0}C.

2.3.2.1 Preparation of Capillary Glass Tubes.

Glass capillary tubes with an internal diameter of 1 mm were soaked as described for the glass plates (Section 2.2.4), with a minor alteration of drying the glass tubes at 37 ^{0}C. The tubes were marked 2 cm from the top and sealed at the bottom with parafilm and pre-warmed for 30 min at 37 ^{0}C just prior to loading the gel mixture. The tubes were supported on a rack supplied by Fisons Plc.

2.3.2.2 Preparation and Loading of Gel Mixture

The first dimension gel mixture of 9.2 M u-p Urea (Fluka), 6.8 % (v/v) NP40, 5 % (w/v) Acrylamide, 0.25 % (w/v) Bisacrylamide, 8.5 % (v/v) Ampholine mix (Pharmacia-Biotech) was incubated at 37 ^{0}C until all the urea had dissolved, at which point the polymerisation agents were added (12 μl 10 % (w/v) ammonium persulphate and10 μl TEMED). The mixture was loaded into the tubes using a 5 ml syringe and needle to a 2 cm mark from the top. 2-D

overlay (6 M Urea) was added (using a 1 ml Hamilton glass syringe) to the top of the gel and left at RT for 1 hr to set. The overlay was then replaced with lysis buffer (9.5 M u-p Urea, 2 % (v/v) NP40, 50 mM DTT, 2 %w/v Ampholines pH 3.5-9.5) and sealed with parafilm. The tubes were normally prepared for electrophoresis a day in advance and stored at RT until required, with the lysis overlay intact.

2.3.2.3 Preparation of Samples for First Dimension Gel Electrophoresis.

50 µl aliquots was defrosted, microcentrifuged for 3 mins at 12, 500 rpm. For every 10 µl aliquot of sample, 8 mg of u-p urea and 20 µl of diluting buffer (9.5 M u-p Urea, 5 % (v/v) NP40, 2 % w/v Ampholines pH 3.5-9.5, 50 mM DTT) was added. The mixture was incubated at 37 ^{0}C to assist the solubilization of the urea. The sample mixture was then ready to be loaded onto a tube gel.

2.3.2.4 Protein Separation by NEPHGE.

The lysis buffer was removed from the top of the tube gels and the samples were loaded (equalised for counts). The sample was then overlaid with 2-D sample overlay (8 M Urea containing 1 % (v/v) Ampholines, pH 3.5-9.5) to ensure entrapment and this was filled to the top of the glass tube. Pre-loaded tube gels were placed into the Shandon Disc Electrophoresis Unit, 1N NaOH and 0.2 M H_2PO_3 buffers were poured into the top and bottom chambers of the unit respectively. Upto 8 tube gels could be run at any one time and a standard run would include at least one tube gel containing marker proteins (Biorad Pre-Mixed 2-D Standards, loaded according to manufacturers instructions). In some instances the markers would be co-electrophoresed with a given sample. Volumes of marker proteins loaded varied for optimisation between 20 - 40 µl. Tube gels were run at 500 V for 6 h (3000 V/ h) at RT.

2.3.2.5 Extraction of Gels and Storage.

Once the tube gels had run to completion, the gels were extracted from the glass tubing by high pressure using a syringe filled with u-p dH_2O attached to one end of the glass tube by rubber tubing. The capillary gel was ejected onto tin foil, and immediately wrapped up and stored at -20 ^{0}C prior to separation in the second dimension.

2.3.3 Electrophoretic Separation of Proteins in the Second Dimension Using Dual Mini-Vertical Gel System.

Proteins were separated in the second dimension on 5 - 15 % gradient (19:1 acrylamide: bisacrylamide, Fisons Plc.) mini-slab gels. The electrophoresis unit used was the dual mini-vertical gel system (Fisons Plc.). The Fisons Phigel 1, acrlyamide/bisacrylamide solution was deionised with Amberlite MB-3 (Sigma) and filtered through Whatman No. 1 prior to use and stored in the dark at 4 ^{0}C.

2.3.3.1 Preparation of Slab Gels.

All the solutions and reagents used for the second dimension run are the same for 1-D SDS-PAGE (Section 2.2.4.1). The gradient gels were cast in batches of 12 and consisted of 5 - 15 % 19:1 acrylamide: bisacrylamide. resolving gradient mix with no stacker.

5-15% Resolving Gel Mixture.

The following stocks were made up in double quantities and stored at 4 ^{0}C.

	Stock	
	5 %	15 %
40 % Acrylamide	3 ml	9 ml
Resolving gel buffer	6 ml	6 ml

dH_2O	14 ml	2.4 ml
Glycerol	-----	3.6 ml

6 ml of each stock gave enough to pour one Fisons min-slab gel. To each solution the following polymerisation agents were added:-

10 % (w/v) ammonium persulphate	50 µl
TEMED	2.5 µl

Both stocks were poured into a Hoefer gradient maker to generate a 5-15 % gradient, which was poured via peristaltic pump set at 15 rpm. The mix was poured to 0.5 cm from the top of the glass plate rim and subsequently overlaid with butanol. For pouring multiple numbers of plates simultaneously, the Mighty Small Multi Pouring stand (Hoefer Scientific Instruments Ltd.) was utilised, where by upto 12 gels (dimensions per gel, 80 mm x70 mm x 0.75 mm) could be poured at any one time, as described by the manufacturer's instructions.

2.3.3.2 Loading of Tube Gels.

The 1:4 running gel buffer was removed from the top of the slab gel and the surface of the gel was briefly washed with equilibration buffer (0.125 M Tris-HCl, pH 6.7, 2.5 mM DTT, 2.3 % w/v SDS). Iodacetemide was added to the equilibration buffer, in an attempt quench excess DTT, which may cause artificial shading at the bottom of 2-D silver stained gel. For every 25 mg of DTT present in the buffer, 450 mg of iodacetemide was added. Modified form Cash et. al., 1995.

The tube gels which had been previously stored at -20 ^{0}C were thawed at RT and gently overlayed onto the gel surface, with the top or basic end of the tube orientated to lie at the left hand side of the slab gel. Once the tubes were pressed down onto the surface of the slab gels, giving close contact with no air bubbles between the interface, hot equilibration buffer

(heated to approximately 60 ^{0}C) was poured and left to incubate at RT for 7 min, after which the buffer was removed prior to embedding in 1% (w/v) agarose (prepared in 0.125M Tris-HCl pH 6.7, 0.1% (w/v) SDS with bromophenol blue) and the slab gels were placed into the electrophoresis unit.

2.3.3.3 Separation of Proteins in the Second Dimension.

The buffer system used was described previously (Section 2.2.4.3) at constant current of 15 mA per gel until the bromophenol blue tracking dye reached the bottom of the slab gel, usually within 1.5 - 2 hrs. During electrophoresis the slab gels were cooled with running water. Following electrophoresis, the gels were stained (Section 2.4) or electrophoretically transferred (Section 2.5) to nitro-cellulose membrane and subsequently visualised.

2.4 Staining and Image Capture of Gels.

2.4.1 Protein Detection.

Gels to be silver stained were fixed (50 % (v/v) Methanol, 12 % (v/v) Glacial Acetic Acid containing 0.5 ml 37 % (v/v) Formaldehyde/ l) for a minimum of 1 hr and subsequent staining was carried out according to the method of Blum et. al. (1987).

2.4.2 Image Capture.

Just prior to drying the gel images were captured using a Sony 3CCD colour video camera (mono setting) based system linked to Quantimet 600 image processing and analysis system (Leica). The captured images were stored as 32 bit tiff files. All photographs of stained and autoradiographic gels presented in the following chapters were taken from images captured using the above system and photographed using Microsoft Windows '95 computer running "PaintShop Pro" software.

2.4.3 Gel Preparation for Drying.

Gels were prepared for drying by incubating overnight in Pre-dry (35 % (v/v) Ethanol, 1.5 % (v/v) Glycerol). Radioactive gels were flourographically enhanced by further soaking in Amplify™ (Amersham) for a maximum of 1 hr.

2.4.4 Drying and Film Development.

Non-radioactive gels were dried in the Easy-Breeze drying unit between sheets of cellophane at the cool setting overnight. After soaking in Amplify the radioactive gels were rinsed briefly

in water prior to vacuam dried onto Whatman 3MM paper on a LKB Bromma Slab Gel Dryer 2003 linked to a Birchover Instruments Ltd. Freeze dryer, for 1hr, after which they were exposed to Hyperfilm (Amersham) and stored at -70 ^{0}C for upto 2 weeks. The autoradiographic film was developed by hand by incubating for 5 mins in 16 % (w/v) Hydroquinone Sodium Carbonate, washing in water for 1 min, fixed in Ilford Hypam rapid fixer for 5 mins as set out by the manufactures instructions and washed extensively under running water. Once dried, the image was captured using the Leica 6000 software.

2.4.5 Calculation of the Molecular Weights of the Protein Bands from 1-D Gels.

The relative mobility (Rf) and optical density of each band was calculated using the Millipore BioImage 1-D analysis system.

2.4.6 Calculation of the Molecular Weights of the Protein Spots from 2-D Gels.

The apparent molecular weights of the proteins were determined by coelectrophoresis of marker gels containing standard marker proteins of known molecular weights. Computer based 2 - D images were initially analysed using the Phoretix 2 - D Lite software package (Non Linear Dynamics Ltd.) with fuller analysis undertaken using Bio Image Intelligent Quantifier Ver 2.5.0 (B. I. Systems Corp.). Spot positions were detected by and the co-ordinates exported into spreadsheets.

2.5 Protein Immunoblotting.

SDS-PAGE separated proteins were electrophoretically transferred using transfer buffer (25mM Tris, 192mM Glycine and 20 % (v/v) Methanol in dH_2O) onto Protran BA 83 Transfer Media (Schleicher and Schuell) using a Milliblot SDE system (Millipore UK, Ltd.), as instructed by the operation manual and run at 2.5 mA per cm^2 of gel. The method used was based on the procedure of Towbin et. al. (1979).

2.5.1 Protein Detection.

In some instances the membranes were cut in half after electrophoretic transfer and one half would be gently rocked for 1 hr at RT with 0.1 % (w/v) Ponceau S in 5 % (v/v) Glacial Acetic Acid to check for efficient protein transfer. This method of protein detection was based on Carbajal et. al. (1986).

2.5.1.1 Preparation of Pre-made Primary and Secondary Antiserum.

The primary antibodies were prepared as directed by the manufacturer's instructions (Stress Gen Biotechnologies Incorp.) and stored in frozen aliquots at -70 ^{0}C. The secondary HRP-linked antibodies were stored in frozen aliquots at -20^{0}C. Both the primary and secondary antibodies were diluted to the appropriate dilution's and stored at -20 ^{0}C until required. The antibody diluent used for preparing the dilution's was 1 % (w/v) Marvel, 0.05 % (v/v) Tween 20 in PBS

The following lists the primary and secondary antibodies used in this study (Refer to Appendix A, A1 for antibody specificity's).

Antibody:-	Produced by:-	Dilution used:-
Rabbit anti-Hsp 70 mycobacterium Y311 polyclonal	Donated by Dr. A. Mehlert (University of Dundee)	1:500
Rabbit anti-GroEL polyclonal	Donated by Dr. A. Mehlert (University of Dundee)	1:500
Rabbit anti-Ubiquitin polyclonal	Stress Gen Biotechnologies Incorp.	1:500
Sheep anti-AMPK polyclonal (anti-aPAN)	Donated by Dr. S. Davies (University of Dundee)	1:4000
Mouse anti-Hsp 60 monoclonal	Stress Gen Biotechnologies Incorp.	1:500
Mouse anti-Hsp 70 monoclonal	Stress Gen Biotechnologies Incorp.	1:500
NRS	SAPU	1:500
HRP anti-rabbit IgG	SAPU, Amersham ECL detection system	1:1000
HRP anti-mouse IgG	SAPU, Amersham ECL detection system	1:1000

Control blots were incubated in NRS in place of the primary serum.

2.5.1.2 Exposure to Primary Antiserum.

Blots were rinsed briefly in PBS and placed in blocking buffer (5 % (w/v) Non-Fat Dried Milk (Marvel-Premium Brands Ltd), 0.25 % (v/v) Tween 20 in PBS) for 1 hr at RT, with gentle agitation to block non-specific protein binding sites. Blots were washed in PBS/Tween 20 (0.25 % (v/v) Tween 20 in PBS) briefly for 4 x 5 mins, after which they were immersed in the appropriate primary antiserum and incubated overnight with gentle agitation at 4 ^{0}C.

2.5.1.3 Exposure to Secondary Antiserum.

Blots were briefly washed in PBS/Tween 20 for a further 4 x 5 min at RT with gentle agitation. The blots were then incubated for 1 hr at RT with the appropriate secondary antiserum. Blots were again washed briefly, 4 x 5 min. For chemiluminescence detection system, recombinant protein G linked recombinant peroxidase linked (Sigma, cat. P8170) was used instead of secondary antibody at a dilution of 1:4000 in PBS and incubated under the same conditions.

2.5.2 Chromogenic Visualisation of Bound Antibodies.

Visualisation of bound antigens was detected using the chromogenic substrate 3, 3'-Diaminobenzadine tera-hydrochloride (DAB) enhanced with Nickel Chloride based on the method of Harlow and Lane (1988). The solution was prepared just prior to the development of the blot (12 mg DAB, 18 ml 50 mM Tris, pH 7.6, 2 ml 0.3 % (w/v) Nickel Chloride). The DAB was dissolved and the solution was filtered through Whatman No. 1. 20 µl of 30 % (v/v) H_2O_2 was added just prior to the addition of the substrate solution to the membrane. A purple/brown reaction product formed within 2 - 3 mins after constant agitation.

Rinsing the blots in PBS/Tween 20 stopped colour development. The blots were dried and stored between 3MM Whatman paper.

2.5.3 Enhanced Chemiluminescence (ECL) Visualisation of Bound Antibodies.

Visualisation of bound antigens by exposure onto Hyperfilm-ECL using ECL detection system (Amersham Int.) as described by the manufacturer instructions.

2.5.4 Image Capture.

The blot images were captured and stored as described in Section 2.4.2.

2.6 Detection of Trehalose and Trehalase Activity in Mycelial Extracts of Bb24 and 12C.

2.6.1 Initial Preparation of Mycelium for Analysis.

Mycelial mats were harvested, immediately snap-frozen in liquid nitrogen after which they freeze-dried overnight. The freeze-dried cultures were weighed out at 4 ^{0}C and approximately 5 mg per culture was used in the preparation of trehalose for analysis. The remainder was prepared for trehalase analysis and stored at -20 ^{0}C until further processing.

2.6.2 Extraction of Trehalose.

Extraction of carbohydrates were obtained by homogenising for approximately 30 sec, the freeze-dried mycelium in 1 ml of ice cold dH_2O with a micro-pestle with Seesand at 4 ^{0}C. The crude homogenate was boiled for 10 min to further disrupt the cells and deactivate any enzymes present. The samples were microcentrifuged for 5 mins at 10, 000 *g*. Samples were stored at -20 ^{0}C until analysis.

2.6.3 Determination of Trehalose by High Performance Liquid Chromatography (HPLC).

Analysis of the sugar content in the prepared samples was achieved by HPLC using a Biorad fermentation column HPX-87H with a flow rate set at 0.6 ml/ min, temperature at 40 ^{0}C and degassed 0.005 M H_2SO_4 as the systems buffer. The amount of trehalose was measured with a Spectra-Physics Isocratic Refractometer Detector (model SP040XR RI) and quantified with a Spectra-Physics Integrator (model SP4270). Prepared samples were loaded into a 1 ml syringe, with the removal of any bubbles. A 0.2 μm Whatman micropore disposable filter was attached to the syringe to remove any residual particle matter as the sample was being

injected into the column loop. The maximum volume the loop could hold was 20 µl. An external standard containing a mixture of D-glucose, sucrose, trehalose and glycogen made up in dH_2O at concentrations of 5, 1, 0.5, 0.25 and 0.1 mg/ were used to calibrate the system.

Alternatively filtered samples were placed in sequence into a multi-well SectraSERIES autosampler AS100 (Thermoseparation Products Inc.) attached to the column allowing semi-automated loading and analysis. Raw data was captured on integrator, emulator and data processing software, Winner on Windows (WOW) v 1.5.2.

2.6.4 Preparation of Trehalase Extracts.

The method of extraction used was based on the method previously described for trehalose extraction, with minor modifications of the following: - 0.5 ml of ice cold 10 mM Mes/KOH, pH 6 (containing 1 mM Benzamadine, 1 mM PMSF, 10 mM NaF), with extraction on ice at 4 ^{0}C. Cell extracts were microcentrifuged at 4 ^{0}C for 5 mins for 10, 000 *g*. 100 µl of the supernatant was used for protein determination and the remainder snap frozen in liquid nitrogen and stored at -20 ^{0}C for further analysis.

2.6.5 Determination of Trehalase Activity.

The enzyme assay used was based on the method of Carrillo et. al. (1994). The assay mixture contained 100 µl of enzyme extract, 100mM trehalose prepared in 10 mM Mes/KOH Buffer and 10 mM Mes/KOH, pH 6 in a final volume of 500 µl was incubated in a 30 ^{0}C water bath for 30 mins, after which the assay mixture was boiled for 2 mins to stop the reaction. The mixture was then microcentrifuged for 10 mins at 3,000 *g* and placed on ice at intermediate stages. Mixtures containing no enzyme extracts were used as blank references.

Glucose liberated in the assay mixture was determined by the glucose oxidase-peroxidase method of Lloyd and Whelan (1969), where for 1 Unit of trehalase released 1 nmol of glucose/min with O.D. values read at 525 nm. The specific trehalase content was calculated against the total protein content present in 100 µl of supernatant.

2.7 Detection of AMP-Activated Protein Kinase (AMPK) Activity.

Dr. S. P. Davies at the Biochemistry Department, University of Dundee, undertook the following experiments in the Laboratory of Prof. G. Hardie.

2.7.1 Sample Preparation.

The harvested mycelium stored under liquid were disrupted in 300 µl of ice cold homogenising buffer B using Seesand on ice at 4 ^{0}C (as described in Section 2.2.12). Extracts were centrifuged (IEF Centra-4R) for 5 mins at 3000 *g* set at 4 ^{0}C and the supernatant aliquots retained and kept under liquid nitrogen until further processing.

2.7.2 Detection of AMP-Protein Kinase Activity Using the SAMS Peptide Assay.

The peptide assay used to detect peptide phosphorylation of AMP-protein kinase activity was carried out by Dr. S. P. Davies as described in Davies et. al., 1989.

2.7.3 Immunological Detection of AMPK.

Immunological studies using the polyclonal alpha-pan AMPK antiserum (raised from purified rat liver AMPK) and control protein to detect the presence of AMPK was kindly donated by Dr. S. P. Davies.

2.8 Expression, Cloning and Sequencing of Putative the Putative Hsp 70 Gene.

2.8.1 DNA/RNA Isolation from *S. lacrymans, S. himantioides* and *Agaricus bisporus.*

2.8.1.1 Reduction of Nuclease Contamination.

All glass and non-disposable plasticware were treated by acid washing in 2% (v/v) Nitric Acid for 24 hrs, followed by rinsing in dH_2O, then sprayed with RNase Free Spray (Promega), re-rinsed in dH_2O and autoclaved for 20 mins. The glassware was then baked at 240 ^{0}C overnight and stored in a nuclease free environment until the next stage of decontamination. Sterile glass- and plasticware were immersed in a solution containing 0.2 % (v/v) diethyl pyrocarbonate (DEPC) - treated dd H_2O, shaken vigorously to dissolve DEPC droplets into solution, pour into 500 ml glass bottles up to the neck and cap, the remaining solution poured into beakers and glass universals and covered with aluminium foil. All containers were left overnight at 37 ^{0}C, followed by autoclaving for 20 mins to remove any residual traces of DEPC, which can carboxymethylate purine residues in the RNA. The glassware was then baked overnight at 240 ^{0}C. Chemicals required for both DNA and RNA extraction were newly obtained from Promega (where otherwise stated) and set aside specifically for use in this work. Two sets of the same chemicals were obtained when necessary, when required for both RNA and DNA preparations thus ensuring no cross contamination between preparations.

Solutions and water were also DEPC treated by making solutions 0.1 % (v/v) with respect to DEPC and incubated overnight at 37 ^{0}C, after which they were autoclaved and stored at RT in a nuclease free environment. Solutions containing Tris buffers were not DEPC treated but prepared with DEPC treated water. Prior to RNA isolation all equipment and work surfaces

were decontaminated with Promega's RNase Free Spray. All work was carried out rapidly on ice to prevent the material from thawing.

2.8.1.2 DNA Isolation.

The DNA extraction protocol was adapted from the method described by Kolar et. al. (1988).

Snap frozen material of *Serpula* species and *A. bisporus* were placed on ice. Approximately 5 - 10 mg of material was used per sample preparation, and placed in a sterile pre-cooled eppendorf tube. Acid washed heat-sterilised Seesand (Fluka) was added to one-tenth the volume of the Eppendorf tube. 400 µl of extraction buffer (0.5 M Tris-HCl, pH 8.5, 0.25 M NaCl and 0.05 M EDTA) with the addition of detergents P-aminosalicyclic acid (PAS) and Tri-isopropynaphthalenesulfonic acid sodium salt (TNS) at a final concentration of 48 mg/ml and 8 mg/ml respectively was used to homogenise the frozen mycelium using a sterile nuclease-free micro-pestle (maximum of approximately 30 sec).

The samples were transferred to a 65 ^{0}C water bath and left for 20 mins. Samples were extracted with 400 µl of phenol: chloroform: isoamyl alcohol, PCIa (24:25:1), shaken and microcentrifuged at 10, 000 *g* for 10 mins at 4 ^{0}C. The top aqueous layer was recovered into a fresh sterile Eppendorf ensuring that no material was removed from the interface, fresh PCIa added and the above procedure repeated twice. An equal volume of chloroform: isoamyl alcohol (24:1) was added to this last aqueous phase, inverted, mixed and microcentrifuged at 10, 000 *g* for 10 mins at 4 ^{0}C. After the phases separated the clear top aqueous phase was recovered into another sterile Eppendorf.

The DNA was precipitated out of this aqueous phase by addition of 1/10th the sample volume of 3M sodium acetate, pH 8 and 2 x the sample volume of absolute ethanol (previously stored

at -20 ^{0}C). The mixture was then inverted gently to mix and stored at -20 ^{0}C overnight. The precipitated DNA was pelleted by microcentrifugation for 10 mins at 10, 000 *g* at 4 ^{0}C, rinsed 2 x with ice cold absolute ethanol. The ethanol was decanted ensuring that the pellet was not dislodged and air-dried on ice. The pellet was resuspended in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA) containing 10 µg/ml RNase A (Sigma, derived from bovine pancreas and free of DNase) to remove residual RNA.

The quality of the DNA was checked on a 2 % agarose gel/ 1 x TBE Buffer (0.54 g Tris, 2.75 g Boric Acid, 0.465 g EDTA, pH 8.2-8.4). The 5 µl of each sample was mixed with 1 µl of loading dye (Promega Blue/orange 6 x dye:- 10 % Ficoll 400, 0.25 % Xylene cyanol FF, O.4 % orange G, 10 mM Tris/ HCl (pH 7.5) and 50 mM EDTA) and subsequently loaded onto the 2 % agarose gel, run at 52 mA, constant voltage for approximately 45 mins. DNA was visualised by ethidium bromide staining (Amresco, 0.625 mg/ml prepared according to manufacturers instructions) incubated with gentle shaking for 10 min, washed for 2 x 5 min and visualised on a UV light box. Picture was taken using a Polaroid camera containing an orange filter with Polaroid 667 black and white instant film.

The prepared DNA was used directly in a PCR reaction or stored at -20 ^{0}C until required. Appropriate dilutions of the DNA samples were made by visual examination of the ethidium bromide gel. Where the brightest bands were of the same intensity as the 500 bp DNA marker standard (approximately 150 ng in 5 µl of 100 bp DNA ladder: Promega) were diluted 1:200 and lesser intense bands in the order of 1:100 and 1:50. A highly concentrated amount of DNA present in the PCR mix effectively inhibited the reaction.

2.8.1.3 Total RNA Extraction.

The RNA extraction protocol was adapted from the procedure of Chomczynski and Sacchi (1987).

Snap frozen mycelium was placed on ice. Approximately 10-50 mg of material was used per sample preparation and placed in a sterile pre-cooled Eppendorf tube. 500 µl of RNA extraction buffer (4 M Guanidinium thiocynate, 25 mM Sodium citrate, 0.5 % (w/v) N-lauroylsarcosine and 0.1 M DTT) and acid washed heat-sterilised Seesand (Fluka-Chemika) was added to one-tenth the volume of the eppendorf tube. The frozen mycelium was homogenised using a sterile DEPC-treated micro-pestle until the mycelium had broken up (maximum of approximately 30 sec).

For every 500 µl of RNA extraction buffer used, the following was added sequentially with thorough mixing after each addition: - 50 µl 2M sodium acetate, pH 4, 500 µl phenol, 100 µl chloroform-isoamyl alcohol mix (49:1). The final suspension was vigorously shaken for approximately 10 sec and incubated for 15 mins on ice.

The resulting solution was microcentrifuged at 10, 000 *g* for 20 mins at 4 ^{0}C The clear aqueous phase containing the RNA was recovered into a fresh sterile eppendorf ensuring that no material was removed from the interface which contained DNA and protein debris. The aqueous phase was mixed with an equal volume of isoproponal (Sigma) and for 1 h at -20 ^{0}C precipitate the RNA.

The RNA was pelleted by microcentrifugation at 10, 000 *g* for 20 min at 4 ^{0}C. The supernatant was carefully discarded and the pellet resuspended in 300 µl of denaturing solution to which 1 volume isopropanol was added and the RNA was allowed to precipitate for 1 h or at -20 ^{0}C.

RNA was pelleted as previously, the supernatant discarded and the pellet was allowed to air dry on ice, and resuspended in 50 µl of nuclease free water (Promega). Removal of contaminating DNA from samples was achieved by incubating the RNA samples with Promega's RQ1 RNase-Free DNase. Preparing a cocktail, in a total volume of 50 µl at a final concentration, per sample as follows: 10 mM NaCl, 6 mM $MgSO_4$, 10 mM $CaCl_2$, 2 µl 1 U/ µl RQ1 RNase-Free DNase, 1.25 µl 40 U/ µl RNasin Ribonuclease Inhibitor, 40 mM 100 mM Tris-HCl, pH 7.9. The cocktail was added to 50 µl of sample, mixed and incubated for 15 mins at 37 ^{0}C. The reaction was stopped by the addition of 2 x volume ice cold absolute ethanol with 1:10 (v/v) 3 M Sodium acetate, pH 5, incubated overnight at -20 ^{0}C, microcentrifuged at 4 ^{0}C for 10 min at 12,500 rpm. The pellet was retained and reconstituted in 50 µl of nuclease-free water (Promega) and stored as previously described.

Long term storage of the RNA was achieved by the addition of 2 x volume ice cold absolute ethanol with 1:30 (v/v) 3 M sodium acetate, pH 5. After long term storage the samples were microcentrifuged at 4 ^{0}C for 10 min at 10, 000 *g*. The pellet was retained and reconstituted in 50 µl of nuclease-free water as described previously. The total RNA yield of each extraction was calculated using a Perkin Elmer UV/Vis Spectrometer, Lambda 2and assuming that a solution of 40 µg/ml total RNA in a 1 cm path possesses an absorbency at 260 nm of 1.0 unit. Each RNA sample (10 µl of sample in a final volume of 1 ml of DEPC-treated HPLC water) was assayed in triplicate at both 260 and 280 nm in quartz quvettes. Chemically pure RNA has an A_{260}/A_{280} of 2.0. Significant deviation from this ratio indicated contamination.

2.8.2 Primer Design from the *A. bisporus* HspA (70) Gene Sequence.

The design of the primers was achieved by access to the bioinformatic resources at Daresbury laboratories (www.seqnet.dl.ac.uk) with the use of the Genetics Computer Group (GCG) sequence retrieval system, scanning the EMBL/GenBank/DDBJ gene databases. The highest percent similarity of known base pair matches between a number of fungi was initiated to search for any known basidiomycete hsp gene sequences. The results from this search gave the basidiomycete *A. bisporus* the highest percentage similarity (Chapter 3, Section 3.3.1). The 20 base pair (bp) primers Nat 1 and Nat 2 were designed from sequence regions before and after an intron (corresponding to nucleotides +247 to +302) found within the partially sequenced 536 bp *A. bisporus* hsp A gene (Schaap et. al. 1996). Amplimers generated from PCR and RT-PCR were 407 bp and 352 bp in size, the difference between the two corresponding to the 50 bp intron spanning the genomic DNA sequence.

The primer sequences were as follows: -

Nat 1 (forward) : 5' CACTCATCTTGGTGGTGAGG 3'

Corresponding to nucleotide +54 to +73.

Nat 1 (reverse) : 5' ATACGGGTGGAACCACCGAC 3'

Corresponding to nucleotides +463 to + 444.

Genosys Biotechnologies Ltd produced the oligonucleotide primers.

2.8.3 Polymerase Chain Reaction (PCR).

PCR amplification was carried out in a reaction mixture containing the following: 2.5 µl of diluted DNA, 1 x Ultrotaq PCR buffer (20 mM Tris-HCl, pH 8.55 containing 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$ and 150 µg/ml BSA), 0.2 mM deoxyribonucleotide triphosphates each (dNTP mix), 50 pmol each of Nat 1 and 2, 0.5 µl of Ultrotaq DNA polymerase (5 U/ µl), reaction mix made up to a final volume of 25 µl with nuclease-free water (Promega). Mineral oil was layered over the reaction mix and amplification was performed using the following conditions on a Techne PHC-3 thermal cycler: 94 ^{0}C (2 min), 94 ^{0}C (15 sec), 60 ^{0}C (30 sec), 72 ^{0}C (1 min). At the end of 30 cycles, the reaction was extended for an additional 7 min. The positive control for an hsp70 product was derived from DNA of the *A. bisporus* mushroom- giving rise to a product 407 bp long. The negative control substituted sterile nuclease-free water for the DNA template in the reaction mixture.

The PCR products were electrophoresed at 52 mA constant voltage for 45 min in a 2 % agarose gel. Molecular weight DNA markers (Promega's 100 bp DNA ladder cat. G2101; Appligene 500 bp ladder cat. 161471) were electrophoresed simultaneously as a reference DNA marker. The gels were stained with 0.5 µg/ml ethidium bromide and visualised under UV light. Pictures were taken of the gels using a Polaroid with 667 black and white film. Enhanced images of the Polaroid's were taken by a Sony 3CCD black and white video camera linked to a heat sensitive thermal printer (Mitsubishi).

2.8.4 Reverse Transcription - Polymerase Chain Reaction (RT-PCR).

The Access RT-PCR System from Promega to generate first strand cDNA template from a specific target RNA transcripts and subsequent amplification in a one step two-enzyme system was used to generate gene fragments. Optimisation of the reaction was achieved by the addition of neat, 1:100 and 1:200 dilution of the prepared RNA in the system. As for DNA, a high concentration of RNA in the mixture inhibits the amplification stage of the reaction.

RT-PCR was carried out in a reaction mixture containing the following: 2.5 µl of 1:200 dilution of RNA, 1 X AMV/ T*fl* reaction buffer, 0.2 mM dNTP mix, 50 pmol each of Nat 1 and 2, 1 mM $MgSO_4$, 0.625 µl of RNasin Ribonuclease Inhibitor (40 U/µl, Promega), 0.5 µl of AMV Reverse Transcriptase (5 U/ µl), 0.5 µl T*fl* DNA Polymerase (5 U/µl), made up in a final volume of 25 µl with nuclease-free water (Promega). Mineral oil was layered over the reaction mix and the RT-PCR performed using the following conditions on a Techne PHC-3 thermal cycler: 48 ^{0}C (45 min), 94 ^{0}C (1 min), 94 ^{0}C (15 sec), 60 ^{0}C (30 sec), 68 ^{0}C (1 min). At the end of 30 cycles, the reaction was extended for an additional 7 min. Positive and negative control reactions were performed to ensure optimisation to the system. For the positive internal control, control RNA with carrier, upstream and downstream control primers provided with the kit were used giving an amplification product of 323 bp long. External positive controls in the form of A. bisporus RNA giving rise to a product of approximately 352 bp long. The negative control was substituted sterile nuclease-free water for the RNA template in the reaction.

The RT-PCR products were electrophoresed and visualised as described in Section 2.9.1.

2.8.5 Cloning and Sequencing of cDNA and Genomic DNA Products.

The following procedures were undertaken in the laboratory of Dr. G. Machray at the Department of Cell and Molecular Genetics at the Scottish Crop Research Institute (S.C.R.I.), Invergowrie, Dundee.

2.8.5.1 Purification of PCR and RT-PCR Products

The desalting and removal of excess primers from the products was achieved by spin-column chromatography. MicroSpin™ S-300 HR Columns from Pharmacia Biotech (cat. XY-047-00-17) were used as described by the manufacturers instructions. The purified samples were collected and ligated to pGEM-T vector (Promega, cat. A13600)

2.8.5.2 Ligation of Purified Products.

The products were ligated using Pharmacia Biotech Ready-To-Go™ T4 DNA Ligase (product no. 27-0361-01) as described by the manufacturer's instructions.

2.8.5.3 Transformation of Competent Cells.

1 µl of ligation reaction was used to transform 100 µl of MAX Efficiency DH5α™ competent cells from GIBCO BRL (cat. 85258SA), as described by the manufacturer's instructions. 100 µl of diluted (1:10) and undiluted resultant cell suspension were inoculated onto pre-made Luria-Bertaini plates (LB plates :- 15 g Tryptone, 5 g Yeast extract, 5 g NaCl, 1 ml 1 M NaOH, 15 g Bacto-agar in 1l dH_2O) containing 100 µg/ml ampicillin. The plates were incubated o/n at 37 ^{0}C, 10 colonies from each were inoculated onto fresh plates and subsequently used as stocks for mini-plasmid DNA preps.

2.8.5.4 Plasmid Preparation and Sequencing.

Liquid stocks of the recombinant colonies were prepared for plasmid DNA preparation. Single colonies were inoculated into 4 ml of pre-made LB containing 50 μg/ml ampicillin and incubated o/n in a 37 ^{0}C orbital incubator. The resultant bacterial cell suspension was pelleted by centrifugation for 5 min at 4, 000 *g* (Hettich Universal Camlab Centrifuge) and the supernatant poured off, retaining the pellet. Isolation and purification of the plasmid DNA was achieved using Promega's Wizard® *Plus* SV Minipreps DNA Purification System, (cat. A1330), spin protocol as described by the manufacturer's instructions. A restriction enzyme digest of the plasmid DNA was done to check that the DNA insert was present. To 8 μl of purified DNA, 1 μl of buffer H and 1 μl of EcoRI (Boehringer Mannheim) were added and incubated for 1 h in a 37 ^{0}C water bath. The resultant digest was analysed on a 1 % agarose gel with Boehringer Mannheim Marker VIII, 100 bp ladder used as reference. The remaining purified DNA was used for sequencing carried out by the sequencing division at S.C.R.I.

2.9 *In Vitro* Translation of *S. lacrymans* and *S. himantioides* mRNA.

2.9.1 Total RNA Extraction.

Total RNA extraction was achieved by the method described previously in Section 2.9.1.4.

2.9.2 mRNA Isolation from Total RNA.

Using total RNA as starting material, small scale mRNA isolation was achieved by using Promega's PolyATract System (cat. Z5310) as described by the manufacturer's instructions. The resultant pellet was resuspended in 10 µl of nuclease free water and stored at -70 ^{0}C until further required.

2.9.3 *In Vitro* Translation Using a Rabbit Reticulocyte Lysate System.

The translation system used was the Rabbit Reticulocyte Lysate System from Amersham Life Science (cat. RPN 3150) and the protocol were set out as described by the manufacturer's instructions. In brief the standard 50 µl translation reaction mixture contained the following: 4 µl 12.5 x translation mix, 2 µl 2.5 M potassium acetate, 1 µl 25 mM magnesium acetate, 5 µl of [^{35}S] L- methionine (37 MBq stock, cat. no. cat. SJ 1015, Amersham International Limited, UK), 10 µl of RNA sample, 20 µl rabbit reticulocyte lysate, made upto a final volume of 50 µl with nuclease free water. The mixture was incubated at 30 ^{0}C for 1.5 h, then placed on ice.

2.9.4 Analysis of Translation Products.

The translation products were analysed by electrophoretic separation as described in section 2.4, with minor modification of the sample preparation whereby the translation product was diluted 1:10 with 2x cracking buffer, boiled for 4 min and microcentrifuged for 3 min at 10, 000 *g*. 10 µl of the denatured sample was subsequently loaded. The separated proteins were silver stained, flourographically enhanced, dried and exposed to X-ray film as described in Section 2.4. The presence of hsps was detected by chemiluminescence enhanced western blotting as described in Sections 2.5.3 and 2.5.4

2.9.5 Incorporation Assay.

To determine the labelled amino acid incorporation into protein, 25 % TCA / 2 % casein acid hydrolysate precipitation was carried out on the translation extracts. 2 µl of lysate mix (samples plus control and blank) was diluted in 250 µl 1N NaOH/ 2 % H_2O_2, incubated at 37 ^{0}C for 10 min. 1 ml of ice-cold 25 % TCA/ 2 % casein acid hydrolysate was added, mixed and left on ice for 30 min. The precipitate was collected by filtering the solution onto a Whatman GF/A glass fibre filter wetted with 5 % TCA. The samples were filtered under vacuam using a Buckner funnel and hand held Whatman vacuam pump. The filters were rinsed initially 3 x 3 ml ice-cold 5 % TCA and finally 1 x 3 ml acetone. The filter discs were dried and transferred into scintillation vials containing 4 mls of Ecosynt A (National Diagnostics). The amount of radioactive counts present was measured by a scintillation counter (LKB Wallace, 1216 Rackbeta Liquid Scintillation Counter linked to a Wilker Digital Decwriter IV) which was programmed to read counts for ^{35}S-methionine. To determine the total amount of counts present, 2.5 µl of translation mix was spotted directly onto a filter and allowed to dry and transferred to scintillation vials as described previously.

CHAPTER THREE

RESULTS

3.1 Metabolic Labelling Studies: Analysis of Heat Induced Changes in Protein and mRNA Synthesis.

3.1.1 Metabolic Labelling Studies Utilising 1-D and 2-D SDS PAGE for the Analysis of Changes in Protein and mRNA Synthesis.

Metabolic labelling studies, incorporating [^{35}S] methionine were used to detect changes in protein expression occurring during exposure to sublethal, extreme and recovery regimes applied to cultures of *S. himantioides* (Bb24) and *S. lacrymans* (12C). Protein extracts were analysed by SDS-PAGE to determine whether the classic heat-shock response occurs for both species. Universally this response involves the rapid but transient reprogramming of cellular activities to ensure survival during the period stress, to protect essential cellular components against heat damage and to permit rapid resumption of normal cellular activities during the recovery period (Burton 1986). The experimental rationale was to investigate the phenomenon of heat-shock induced thermotolerance in cultures of Bb24 and 12C by monitoring the organisms ability to recover from an otherwise extreme heat-treatment respectively. This was illustrated primarily by overall changes in protein synthesis and the production of specific proteins of the hsp families associated with this response.

The aims of the work described in this section were to determine the following points.

(i) Whether the sublethal training regimes (Train A and B) used for Bb24 and 12C, effectively induced thermotolerance with respect to the ability to survive otherwise extreme heat-treatments, by reactivation of protein synthesis during the recovery periods. This was achieved by monitoring overall changes in protein synthesis i.e. whether the relationship of

heat-induced inhibition of protein synthesis (HIIPS) and thermotolerance could be correlated with the ability to survive these extreme heat-treatments.

(ii) To identify changes in 1D and 2D protein profiles and specific identification of proteins, which could be related, by molecular weight, to important hsps.

(iii) Whether, by *in vitro* translation studies, any changes in mRNA expression could be correlated by molecular weight to specific precursors for hsps.

3.1.2 Effect of Heat Treatments on Heat Induced Inhibition of Protein Synthesis (HIIPS).

One aspect of the molecular response of Bb24 and 12C to elevated temperatures may be observed by assessing the overall levels of protein synthesis. The level of synthesis and its inhibition was examined in two ways: by means of incorporation of [^{35}S] methionine into TCA-precipitated material (Table 3.1.0) and 1D SDS-PAGE analysis (Figure 3.1 A, B, C and D) of labelled cultures (refer to Chapter Two, Section 2.1.1.3 for labelling periods).

Treatment Regimes		Mean Incorporation of [^{35}S] methionine (dpm)	
		Bb24	12C
Train C	(Tr C)	151423	154749
	(Tr C/HS)	4761	13844
	(Tr C/HS/R)	147364	1745
Train A	(Tr A)	17585	19264
	(Tr A/HS)	45214	7612
	(Tr A/HS/R)	122253	1882
Train B	(Tr B)	115890	179232
	(Tr B/HS)	82839	45610
	(Tr B/HS/R)	105790	1713

Table 3.1.0. Mean incorporation of radioactivity into nascent proteins were determined by TCA precipitation and scintillation counting. The data represents the average of two labelling experiments.

The ratio of the mean percentage incorporation in heat-treated cultures to that found in their non-heated control counterparts was taken as a measure of the relative protein synthesis (RPS). Values for heat induced inhibition of protein synthesis (HIIPS) were calculated by subtracting from 1.0 the value of relative protein synthesis (Laszlo 1988), and therefore HIIPS = 1 - RPS (Table 3.1.1).

Treatment Regimes		Bb24		12C	
		RPS	HIIPS	RPS	HIIPS
Train C	(Tr C)	1.000	0.000	1.000	0.000
	(Tr C/HS)	0.030	0.970	0.087	0.913
	(Tr C/HS/R)	0.970	0.030	0.011	0.989
Train A	(Tr A)	0.116	0.844	0.122	0.878
	(Tr A/HS)	0.298	0.702	0.048	0.952
	(Tr A/HS/R)	0.807	0.193	0.012	0.988
Train B	(Tr B)	0.765	0.235	1.138	-0.138
	(Tr B/HS)	0.547	0.453	0.289	0.710
	(Tr B/HS/R)	0.689	0.302	0.011	0.989

Table 3.1.1. Relative protein synthesis (RPS) and heat induced inhibition of protein synthesis (HIIPS) in intracellular extracts of Bb24 and 12C cultures exposed to various temperature regimes.

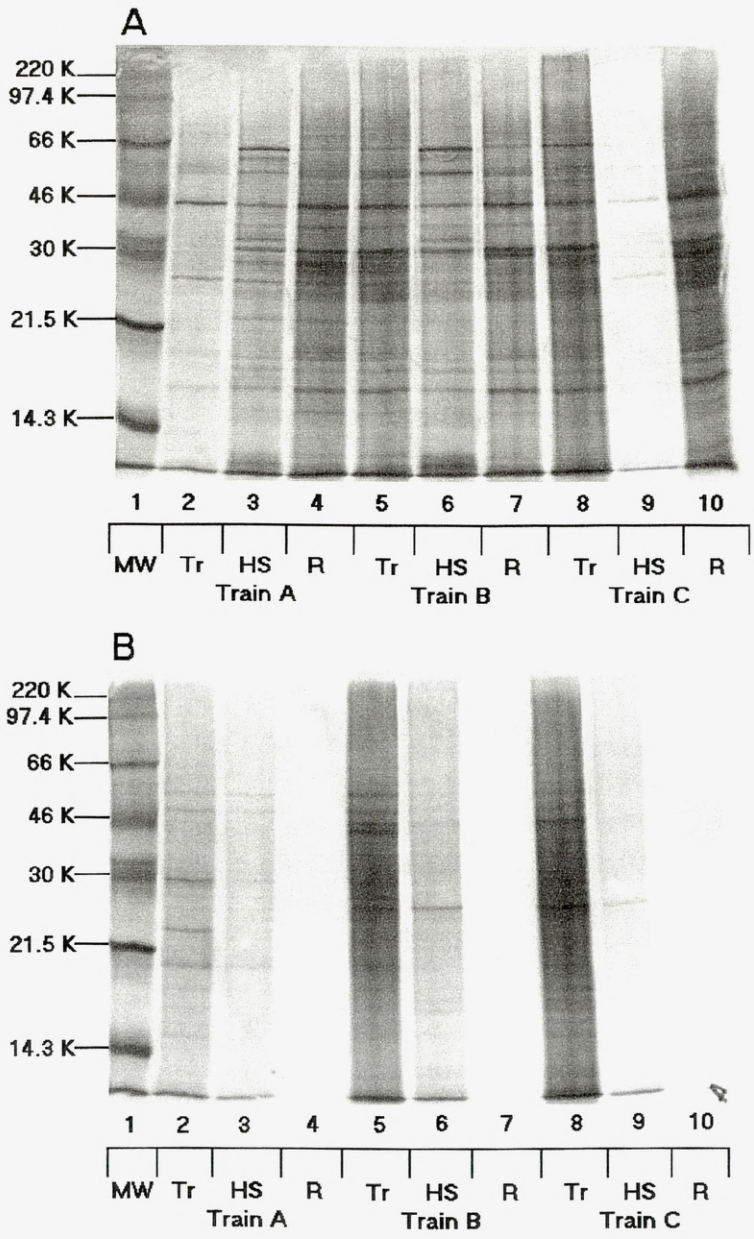
Visual inspection of the autoradiograms illustrated in Figure 3.1 A, B, and C, D of [^{35}S] methionine labelled extracts of Bb24 and 12C respectively, leads to several impressions concerning the effect of the various heat treatment regimes upon levels of protein synthesis. In the first instance, extreme heat treatment leads to inhibition of protein synthesis in the control (Train C) cultures of both Bb24 and 12C (lane 9 - Tr C/HS) respectively, with a considerable marked reduction in bands present when comparing to the respective control profiles (lane 8 - Tr C).

In comparison, Bb24 cultures (Figures 3.1 A and C) show a marked increase in the level of synthesis during the recovery period of extreme heat-treated control cultures (lane 10 - Tr C/HS/R). This is illustrated by comparison of the recovery profile to the untreated control

(lane 8 - Tr C) counterpart, which it closely resembles. This observations leads to the conclusion that 40 ^{0}C /3 hr does not effectively kill Bb24 mycelium which was originally presumed in studies of White et. al. (1995).

There is a difference in labelled profiles between Bb24 Train A and B cultures to which the degree of HIIPS appears to be greater in Train A profile (lane 2) illustrated by the reduction in the number and intensity of bands compared to that of Train B profile (lane 5).

A similar trend in the level of protein synthesis is observed in 12C extreme heat-treated Train A and B cultures (Figures 3.1 B and D, lanes 2 and 5 respectively). However there is a clear indication that during recovery, protein synthesis is markedly reduced and the level of inhibition is at its highest, with an absence of bands in both the treated and untreated 12C cultures (lanes 4, 7 and 10).



Figures 3.1 A and **B**. Metabolic labelling experiment, autoradiograms of [^{35}S] methionine-labelled (A) Bb24 and (B) 12C intracellular proteins synthesised during the different temperature regimes. Values on the left represent molecular weight markers in the range 220 - 14.3 kD. All samples were equalised for protein content of approximately 6 μg and separated in 12.5% SDS polyacrylamide gel.

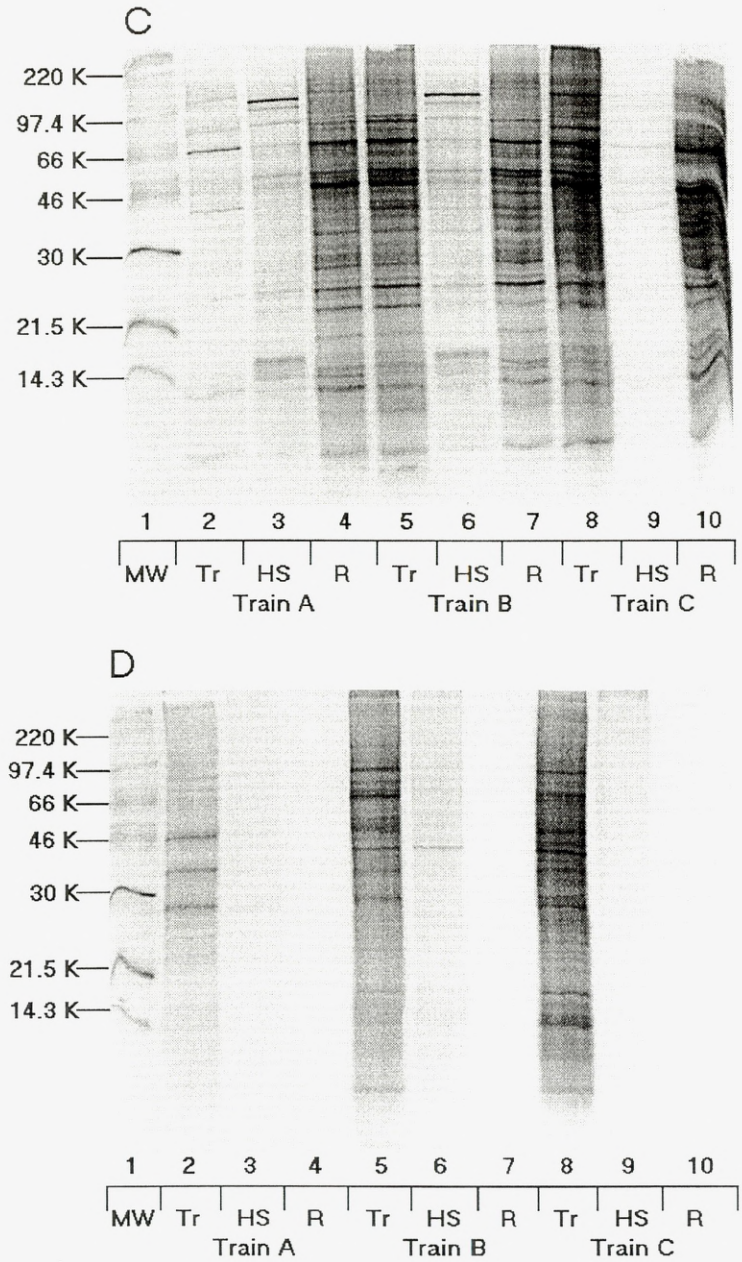


Figure 3.1 C and **D**. Repeat metabolic labelling experiment, autoradiogram of [^{35}S] methionine-labelled (C) Bb24 and (D) 12C intracellular proteins synthesised during the different temperature regimes. Values on the left represent molecular weight markers in the range 14.3 - 220 kD. All samples were equalised for protein content of approximately 6 µg and separated in 12.5% SDS polyacrylamide gel.

Figure 3.1 A and **C**, represents Bb24 labelled intracellular extracts from cultures which have undergone various temperature regimes. Lane 1 (MW) - rainbow high molecular weight markers in the range of 14.3 - 220 kD (Amersham International plc.).

Lanes 2 - 4 represent Train A cultures (28 ^{0}C/ 48 hr):

lane 2 (Tr) - 28 ^{0}C/ 48 hr;

lane 3 (HS) - exposed to extreme heat treatment, 40 ^{0}C/ 3 hr;

lane 4 (R) - placed into recovery at optimum condition's 25 ^{0}C/ 24 hr.

Lanes 5 - 7 represent Train B cultures (30 ^{0}C/ 48 hr):

lane 5 (Tr) - 30 ^{0}C/ 48 hr;

lane 6 (HS) - exposed to 40 ^{0}C/ 3 hr;

lane 7 (R) - placed into recovery at 25 ^{0}C/ 24 hr.

Lanes 8 - 10 represent Train C control cultures (25 ^{0}C/ 24 hr):

lane 8 (Tr) - optimum condition's 25 ^{0}C/ 24 hr;

lane 9 (HS) - exposed to 40 ^{0}C/ 3 hr;

lane 10 (R) - placed into recovery at 25 ^{0}C/ 24 hr.

Figure 3.2 B and **D**, 12C labelled intracellular extracts from cultures which have undergone various temperature regimes. Lane 1 (MW) - rainbow high molecular weight markers in the range of 14.3 - 220 kD (Amersham International plc.).

Lanes 2 - 4 represent Train A cultures (28 ^{0}C/ 24 hr):

lane 2 (Tr) - 28 ^{0}C/ 24 hr;

lane 3 (HS) - exposed to extreme heat treatment, 40 ^{0}C/ 2 hr;

lane 4 (R) - placed into recovery at optimum condition's 22 ^{0}C/ 24 hr.

Lanes 5 - 7 represent Train B cultures (25 ^{0}C/ 48 hr):

lane 5 (Tr) - 25 ^{0}C/ 48 hr;

lane 6 (HS) - exposed to 40 ^{0}C/ 2 hr;

lane 7 (R) - placed into recovery at 22 ^{0}C/ 24 hr.

Lanes 8 - 10 represent Train C control cultures (22 ^{0}C/ 24 hr):

lane 8 (Tr) - optimum conditions of 22 ^{0}C/ 24 hr;

lane 9 (HS) - exposed 40 ^{0}C/ 2 hr;

lane 10 (R) - placed into recovery at 22 ^{0}C/ 24 hr.

These visual impressions are confirmed by the quantitative analysis of protein synthesis in relation to HIIPS summarised in Table 3.1.1 illustrated in Figure 3.2.

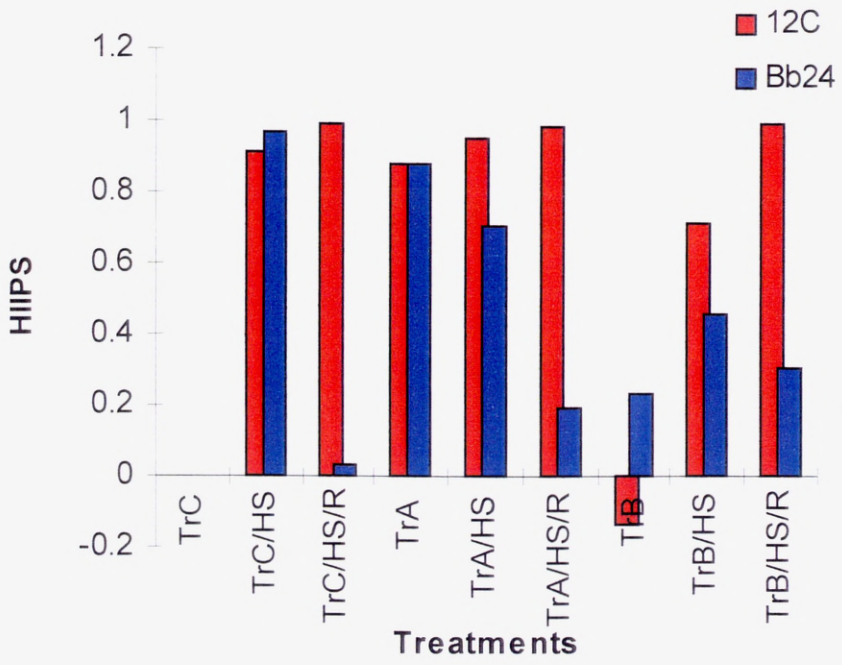


Figure 3.2. The effect of exposure of Bb24 ( ) and 12C ( )cultures, respectively to various heat treatments on heat-induced inhibition of protein synthesis (HIIPS). Bb24 control cultures (Tr C) grown at optimum conditions at 25 ^{0}C for 24 h, were exposed to 40 ^{0}C for 3 h (Tr C/HS) and allowed to recover at 25 ^{0}C for a further 24 h (Tr C/HS/R). Control colonies were trained at sublethal temperature regimes of 28 ^{0}C for 48 hr and 30 ^{0}C for 48 hr (Tr A and Tr B, respectively). Exposed to a extreme heat-treatment of 40 ^{0}C for 3 h (Tr A/HS and Tr B/HS) and allowed to recover at optimum conditions of 25 ^{0}C for a further 24 h (Tr A/HS/R and Tr B/HS/R). 12C control cultures (Tr C) grown at optimum conditions at 22 ^{0}C for 24 h were exposed to extreme heat treatment of 40 ^{0}C for 2 h (Tr C/HS) and allowed to recover at optimum conditions of 22 ^{0}C for a further 24 h (Tr C/HS/R). Control colonies were trained at sublethal temperature regimes of 28 ^{0}C for 24 hr and 25 ^{0}C for 48 hr (Tr A and Tr B, respectively). Exposed to a extreme heat-treatment of 40 ^{0}C for 2 h (Tr A/HS and Tr B/HS) and allowed to recover at optimum conditions of 22 ^{0}C for a further 24 h (Tr A/HS/R and Tr B/HS/R).

In cultures previously exposed to sublethal heat treatment (Train A) for both species, the HIIPS resulting from this exposure was high, 0.884 and 0.878 (Bb24 and 12C respectively, values presented in Table 3.1.1). In comparison, Train B cultures had lower values for HIIPS, 0.235 and -0.138, where the latter training effectively increased the level of protein synthesis higher than that of the 12C control cultures (Train C).

The effect of sublethal heat treatments upon protection of protein synthesis during exposure to extreme heat treatment was different for both Bb24 and 12C respectively. The level of HIIPS observed in Bb24 Train B extreme heat-treated cultures was approximately 1.5 times lower compared to 12C counterparts. While for Train culture HIIPS values were 0.702 and 0.952 for Bb24 and 12C respectively.

When control cultures (Train C) of Bb24 and 12C were exposed to extreme heat treatment respectively, there was a clear reduction in the overall rate of protein synthesis, with a high level of inhibition occurring, corresponding to HIIPS values of 0.97 and 0.913 respectively. There was a distinct difference in the recovery of Bb24 and 12C control cultures after 24 hr in their respective optimum conditions as the level of HIIPS for Bb24 was negligible, 0.03, where as 12C had a value of 0.989. This clearly illustrates the possible thermotolerant and thermosensitive natures of both respective species. Figure 3.2 illustrates this distinct difference between the two species (depicted as Tr C, Tr C/HS, Tr C/HS/R columns for both species). The complete recovery in control Bb24 cultures also demonstrates that the previous observation that 40 ^{0}C for 3 hr does not effectively kill mycelium with resumption of normal cellular activities after 24 hr in recovery (White et. al. 1995).

Recovery of extreme heat-treated, trained cultures for Bb24 and 12C, were again distinctly different. The level of inhibition was markedly reduced in Bb24 cultures, 0.311 and 0.193

(Train A and B respectively) compared to that of 12C cultures where the values for both Train A and Train B, 0.988 were equivalent to that of the control recovery, a value of 0.989. This is clearly illustrated in Figure 3.2 depicted as Tr A/HS/R, Tr B/HS/R and Tr C/HS/R for recovery of extreme heat-treated Train A, B and C cultures respectively.

The observations from the above studies indicate strongly that sustained protein synthesis during extreme heat treatment is dependent on pre-conditioning cultures to sublethal training regimes, for Bb24 cultures only. The sublethal training regimes used on 12C did not effectively induce thermotolerance, as the relative protein synthesis during recovery was negligible. The following points outline the major observations made.

(i) The relationship of heat-induced inhibition of protein synthesis (HIIPS) and thermotolerance can be correlated with the transient ability to survive during extreme heat treatments for Bb24 cultures exposed to sublethal temperatures prior to extreme treatment. This is clearly illustrated in Figure 3.2 by continued synthesis during extreme heat treatment for Train A and B cultures (Tr A/HS and Tr B/HS respectively) compared to the control extreme heat treated culture (Tr C/HS).

(ii) In control cultures protein synthesis is reduced in the extreme heat treatment for both Bb24 and 12C, but after 24 hr in recovery, resumption of normal cellular activities occurred for Bb24 only (as illustrated by the reactivation of protein synthesis), demonstrating that the extreme heat regime of 40 ^{0}C for 3 hr does not effectively kill the mycelium as indicated in previous studies (White et. al. 1995). These observations also clearly demonstrate the overall thermotolerant nature of *S. himantioides* to that of *S. lacrymans*.

3.1.3 Effect of Heat Treatments on Protein Synthesis: Identification of Elevated and Newly Synthesised Proteins by 1-D SDS PAGE.

Following the respective control (Train C) and sublethal (Train A and B) temperature regimes upon extreme, heat and recovery phases on Bb24 and 12C cultures, a complex pattern of protein synthesis became discernible, when analysed by 1-D SDS-PAGE (Figure 3.1 A, B, C and D). Full analysis of labelled profiles was achieved using the Millipore BioImage *Whole Band Analyser* (WBA) software system.

A comprehensive study of the bands detected was carried out to determine the Rf and molecular weight values. The intensity of the bands was measured by integrated optical density (IOD), to give a quantifiable measurement of a band in any given lane as a percentage of the total IOD for a given lane (%IOD). Changes in the %IOD could then be taken to represent the level of synthesis for a particular protein in relation to the various temperature regimes imposed. As all lanes presented in the autoradiograms were normalised for protein content, any changes in band intensity could be attributed to an increase or decrease in synthesis. The autoradiographic profiles of Bb24 and 12C in Figure 3.1 (A and C, B and D respectively) were almost identical when comparing the number and position of bands present in the two sets. Further analysis was therefore concentrated on the autoradiograms illustrated in Figure 3.1, A and B.

By using the Rf measurements obtained from the whole band analysis output files, Figure 3.1 A and B autoradiograms could be summarised diagrammatically (Figure 3.3 A and B) using a computer programme developed for this purpose (programme kindly donated by Mr. P. Smith, at the Scottish Crop Research Institute, Invergowrie, Dundee). The computer application

spatially distributes the Rf values as a series of lines, each line representing a band in descending order of smallest to largest value.

Systematic band comparisons of all the lanes presented in the autoradiograms was also achieved using Millipore's WBA Band Match. The data was cross-referenced with the graphically presented gels in Figure 3.3 to give a complete and accurate picture of important groups of proteins. Constitutively and newly synthesised proteins in the different temperature regimes imposed were highlighted to illustrate proteins related by molecular weight to hsps. Comparisons were made not just within a gel but global matches between gels were achieved to determine the presence of proteins, which were common to both species of *Serpula*.

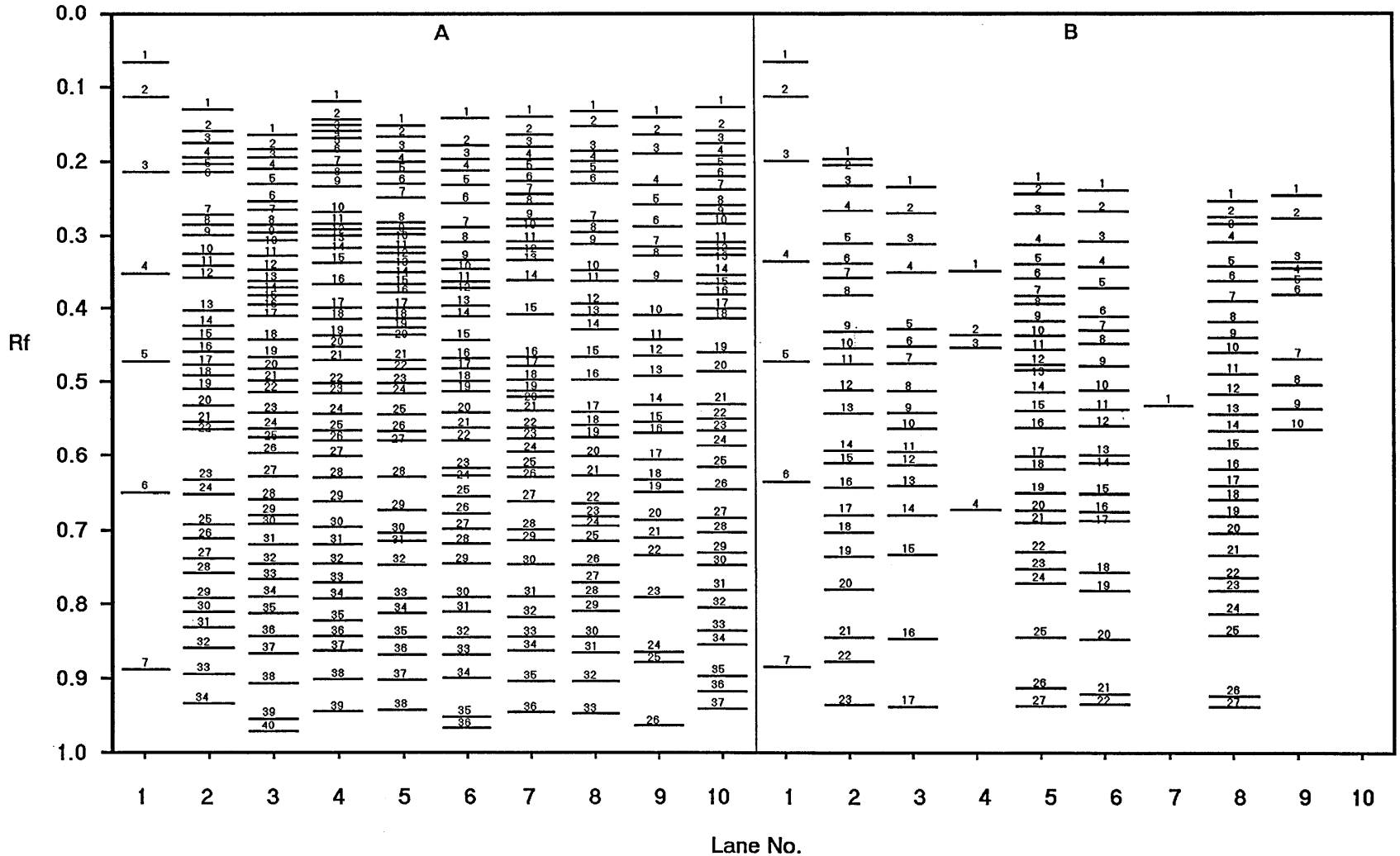


Figure 3.3 A and **B**. Diagrammatic interpretation of the band distribution in autoradiograms illustrated in Figure 3.1 A and B. Numbered lines refer to band numbers (#) on whole band analysis data. Refer to Figure 3.1 A and B legend for lane descriptions.

Tables 3.1.2 and 3.1.3 represents a summary of bands present in extracts of Bb24 which are conserved and newly synthesised respectively, in relation to the control (lane 8 of the autoradiogram depicted in Figure 3.1 A and diagrammatic interpretation in Figure 3.3 A).

Molecular	Lanes								
Weight(kD)	2	3	4	5	6	7	8	9	10
90	1						1		1
83.4			3	1			2		
73.8		2	6	3			3		
69.6	5	4	7	4	3	4	4		4
66.3	6		8	5	4	5	5		5
63		5	9	6	5	6	6	4	6
54.5	7	8	11	8		9	7		9
52.5	8	9	13	10			8		10
50.3	9	10	14	11	8	11	9		11
45.6	11	12		14	10		10		
*42.6	12	13	16	15	11	14	11	9	15
37.9		16	17	17	13		12		17
*36	13	17	18	18	14	15	13	10	18
34	14			19			14		
*30	16	19	21	21	16	16	15	12	19
*28	18	21	22	23	18	18	16	13	20
*25.7	20	23	24	25	20	21	17	14	21
*24.7	21	24	25	26	21	22	18	15	22
24		25	26	27	22	23	19	16	23
23		26	27				20	17	
*21.7	23	27	28	28	24	26	21	18	25
20.6		28	29	29		27	22		
20		29			26		23	20	27
19.3	25	30	30				24		
*18.7	26	31	31	31	28	29	25	21	28
*17.7	27	32	32	32	29	30	26	22	29
16.8	28	33	33				27		
*16.3	29	34	34	33	30	31	28	23	31
15.8	30	35		34	31		29		32
14.8		36	36	35	32	33	30		33
*14.3	32	37	37	36	33	34	31	24	34
12			38	37		35	32		35
9.2	34		39	38		36	33		36

Table 3.1.2. Molecular weights of conserved proteins expressed in Bb24 extracts. Band numbers correspond to those indicated in Figure 3.3 A. Molecular weights prefixed with '*' relate proteins continuously expressed throughout the temperature regimes imposed, illustrated in Figure 3.4 A.

Molecular Weight (kD)	Lanes								
	2	3	4	5	6	7	8	9	10
94.8			1						
***86.2**			2		1	1		1	
81.3			4						
***79**	2	1	5	2		2		2	2
71.1	4	3						3	
60.4						7			
***59**		6		7	6	8		5	8
***57**		7	10						9
***53.4**		9	12	9	7	10		6	
48.6				12		12			
***46.8**	10	11	15	13	9			8	13
41.3		14			12				
40.3		15		16					
33			19	20					
***32.4**	15	18			15			11	
31.4			20						
29.1	17	20		22	17	17			
***27.2**	19	22	23	24	19	19			
26.7						20			
22.2					23	25			
20.8	24				25			19	26
13.5								25	
***8.6**		40			36			26	

Table 3.1.3. Molecular weights of newly synthesised proteins expressed in Bb24 extracts. Band numbers correspond to those indicated in Figures 3.3 A. Molecular weights prefixed with '*' relate to proteins illustrated in Figure 3.4 B.

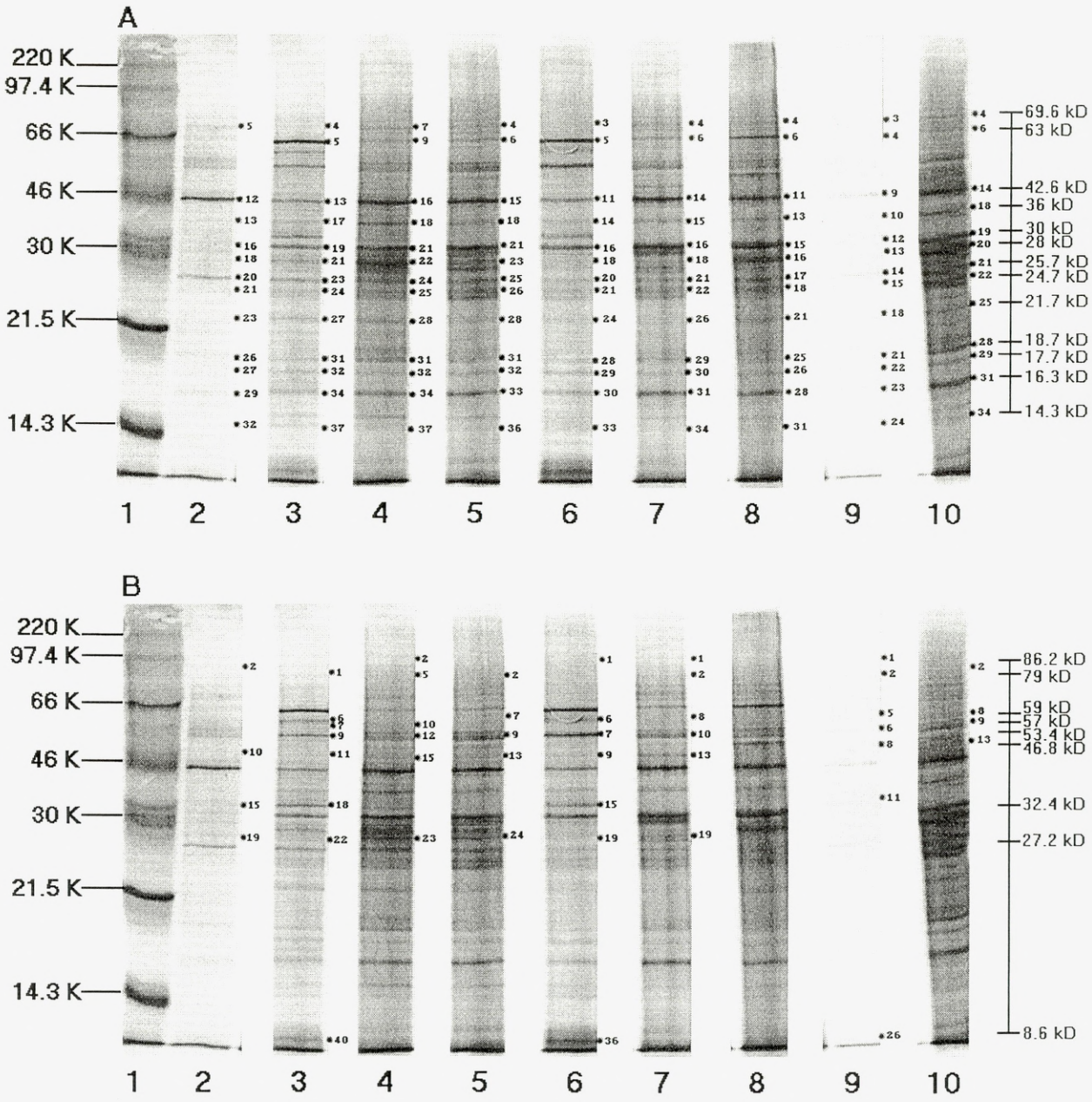


Figure 3.4 A and **B**. Autoradiograms annotated for conserved (A) and newly synthesised (B) proteins present in Bb24 extracts which have undergone various temperature regimes. Values to the left refer to molecular weight markers, 14.3 – 220 kD, and values to the right of the gels represent approximate molecular weights of highlighted bands. Refer to Figure 3.3 A legend for description of lanes 1 - 10 in both profiles.

A core set of proteins which are constitutively expressed throughout the various temperature regimes imposed on Bb24 cultures are within the mid to low molecular weight range of 42.6, to 14.3 kD (prefixed with * in Table 3.1.2, illustrated in Figure 3.4 A). The autoradiogram profile for Bb24 intracellular extracts show a variety of bands which correspond by molecular weight to several hsps: hsp90 (90 kD), 70 (69.6 kD), 30 (30 kD), 26 (25.7 kD), 23 (23 kD), 18 (17.7 kD), 17 (16.8 kD), 16 (16.3 kD) and 12 (12 kD) molecular weights, highlighted in brackets. Within this range, 16. 3 to 30 kD are seen to be constitutively expressed throughout the temperature regimes imposed and could be related by M_r to the small hsp family (M_r of 16 - 40 kD). Another stress related protein running at 63 kD is similar in molecular weight to that of the stress-related AMP-activated protein kinase. The biochemical characterisation of this kinase in rat liver illustrates a single polypeptide chain of 63 kD which contains both the allosteric (AMP) and catalytic (ATP) sites (Carling et. al. 1987).

Changes in the intensity of a particular band in relation to its synthesis can be deduced from the % IOD values. There are distinct differences in the pattern of synthesis when comparing against the various temperature regimes imposed. Table 3.1.4 outlines the % IOD values for the highlighted conserved proteins (prefixed with *) illustrated in Table 3.1.2 and Figure 3.4 A. There is increased synthesis of 36 kD when Bb24 cultures are placed into sublethal temperatures, Train A and B (bands 13 and 18, lane 2 and 5 respectively), with values of 1.16 and 1.37 when compared to the control value of 0.96 (Train C, lane 8, bands 13).

A similar trend can be seen for both 16.3 kD and 42.6 kD proteins present in the control (Train C). With values of 5.25 and 5.84 (Train C, lane 8, bands 28 and 11 respectively), approximately 1.5 and 5.5 fold increase for Train A counterparts (lane 2, bands 29 and 12 respectively); and approximately 1 and 1.6 fold increase for Train B (lane 5, bands 15 and 33 respectively). The opposite of this trend can be illustrated with decreased synthesis during

sublethal temperature regimes (Train A and B) for 69.6, 50.3, 30, 28, 24.7 and 14.3 kD when compared to the %IOD values for their control counterparts. The comparison of %IOD values for Train A and B cultures (lanes 2 and 5 respectively) to control (lane 8) are illustrated in Table 3.1.4, prefixed with $^{+}$ or $^{=}$.

Molecular Weight	Lanes %IOD								
(kD)	2	3	4	5	6	7	8	9	10
*69.6	$^{-}$1.16	0.79	2.07	$^{-}$1.63	0.35	2.70	3.35		3.11
*63		11.83	1.41	$^{-}$1.62	8.34	2.74	6.27	6.08	2.50
*50	$^{-}$0.84	0.54	0.44	$^{-}$2.29	1.19	1.01	3.01		2.10
*42.6	$^{+}$32.0	5.22	8.99	$^{+}$9.23	4.74	10.06	5.84	16.8	2.49
*36	$^{+}$1.16	2.18	4.42	$^{+}$2.37	2.03	3.06	0.96	0.20	1.43
*30	$^{-}$2.01	7.63	8.85	$^{-}$8.85	8.25	11.06	11.06	4.54	5.71
*28	$^{-}$0.96	3.58	13.04	$^{-}$5.26	0.80	2.09	12.36	4.30	9.21
*25.7	$^{+}$13.5	4.39	4.99	$^{-}$5.21	2.62	3.51	5.42	10.84	4.89
*24.7	$^{-}$2.04	2.48	3.17	$^{-}$3.95	2.89	4.49	5.02	3.71	2.72
*21.7	$^{-}$1.34	3.04	1.89	$^{-}$3.04	1.49	1.25	2.80	1.93	1.53
*18.7	$^{+}$4.32	2.37	2.48	$^{-}$2.14	2.33	3.79	2.31	1.88	2.81
*17.7	$^{-}$0.59	2.53	1.11	$^{+}$2.03	3.18	2.10	1.84	5.08	1.82
*16.3	$^{+}$7.85	4.47	4.62	$^{+}$5.69	3.84	7.75	5.25	4.23	5.59
*14.3	$^{-}$0.27	1.65	1.37	$^{-}$0.78	1.66	0.35	1.48	1.99	2.38

Table 3.1.4. Quantification of band intensities measured as % IOD of conserved proteins, which are expressed in extracts of Bb24. Molecular weights prefixed with * relate to proteins illustrate in Figure 3.4 A. Values in lanes 2 and 5 prefixed with $^{+}$ or $^{-}$ correspond to increased or decreased synthesis in relation to control values in lane 8.

One protein, 17.7 kD has increased synthesis during extreme heat treatment, but decreases in synthesis when cultures are placed back into recovery (optimum conditions). The M_r of this polypeptide corresponds to the small heat-shock protein, hsp18. A 4.3 fold increase in this protein, during extreme heat treatment occurs for Train A culture compared to that of Train B and control cultures with a 1.6 and 2.8 fold increase respectively.

Comparisons were also made to identify any newly synthesised proteins which are expressed due to shifts in temperature and which were not present in the control, Train C extracts. These

new proteins ranged from 86.2 to 8.6 kD and are summarised in Table 3.1.3. Proteins of particular interest are those similar in size to hsps illustrated in Figure 3.4 B and include 79, 59 and 27.2 kD corresponding by M_r to hsp80, 60 and 27, respectively. Increased synthesis of a 59 kD protein occurs during the extreme heat treatment of Train A, B and control extracts (lane 3, 6 and 9 in Figure 3.4 B respectively). The highest % IOD was found for the extreme heat-treated control with a value of 4.38. The reverse was observed for 32.4 kD upon extreme heat treatment of Train A and B cultures, the values are 5.81 and 4.08 respectively. This is between 10 to 12 times greater than the extreme heat-treated control (lane 9), with a value of 0.45.

The trend whereby increased synthesis of a particular protein does not occur on exposure to extreme heat treatment is seen for 27.2 kD. The % IOD is higher in both training regimes and recovery periods (lanes 2, 5 and lanes 4, 7 respectively). The reverse of this is illustrated by comparing the % IOD's for the 86.2 kD protein. This is present in low levels in lane 4, 6 and 7 (bands 2, 1, 1 and 1 respectively), but showing increased synthesis in the control culture exposed to extreme heat treatment (lane 9, band 1). The values for these observations are illustrated in the Table 3.1.5.

Molecular Weight(kD)	Lanes %IOD								
	2	3	4	5	6	7	8	9	10
*86.2			0.47		0.97	0.35		2.36	
*79	0.34	0.65	1.46	1.15		0.79		2.80	2.18
*59		3.80		0.26	2.35	1.04		4.34	2.44
*57		0.65	1.25						2.14
*46.8	0.52	1.02	0.98	1.70	1.86	2.48		0.73	1.35
*32.4	1.82	5.81			4.08			0.45	
*27.2	3.75	1.33	7.72	4.44	1.54	3.01			

Table 3.1.5. Quantification of band intensities measured as % IOD of newly synthesised proteins which are expressed during a shift in temperature in extracts of Bb24. Molecular weights prefixed with * relate to proteins illustrate in Figure 3.4 B.

Tables 3.1.6 and 3.1.7 demonstrate comparisons of 12C extracts represented in the autoradiogram in Figure 3.2 B which is graphically interpreted in Figure 3.3 B to identify constitutively and newly synthesised proteins in relation to the control (lane 8 of both Figures) are illustrated in Tables 3.1.6 and 3.1.7.

Molecular Weight (kD)	Lanes								
	2	3	4	5	6	7	8	9	10
*60.6	3	1		2	1		1		
*56.3	4	2		3	2		2	2	
55.6							3		
*50	5	3		4	3		4		
*45	6	4	1	5	4		5	3	
*43.5	7			6			6	5	
41.2				8			7	6	
36.9				9	6		8		
33.7	9	5	2	10	7		9		
31.8	10	6	3	11	8		10		
*30	11	7		12	9		11	7	
*27.7	12	8		14	10		12	8	
*26.4	13	9		15	11	1	13	9	
25.1		10		16	12		14	10	
*23.4	14	11			13		15		
22.4	15	12		18	14		16		
21.4	16	13		19	15		17		
21							18		
*20.3	17	14	4	20	16		19		
19.3	18						20		
18.5	19	15		22			21		
17.8					18		22		
17.2	20			24	19		23		
16.4							24		
*15.2	21	16		25	20		25		
12.1				26	21		26		
*10.5	23	17		27	22		27		

Table 3.1.6. Molecular weights of conserved proteins expressed in 12C extracts. Band numbers correspond to those indicated in Figure 3.3 B. Molecular weights prefixed with '*' relate to proteins illustrated in Figure 3.5 A.

Molecular Weight(kD)	Lanes								
	2	3	4	5	6	7	8	9	10
66.7	1								
65.2	2								
61.8				1					
48								4	
41.9					5				
40	8			7					
29.5				13					
26.9						1			
19.8				21	17				
14.4	22								

Table 3.1.7. Molecular weights of newly synthesised proteins expressed in 12C extracts. Band numbers correspond to those indicated in Figures 3.3 and 3.5 B.

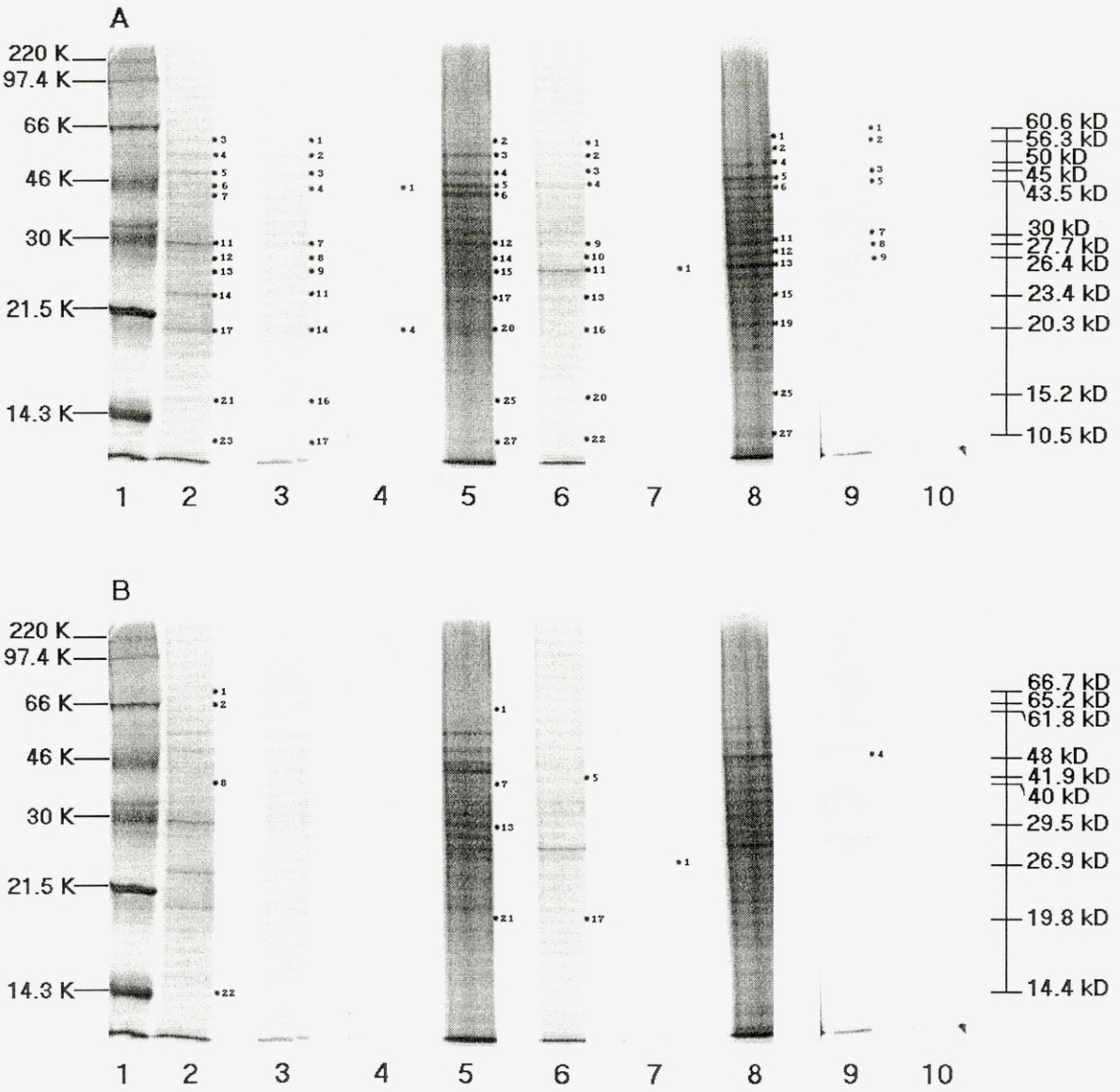


Figure 3.5 A and **B**. Autoradiograms annotated for conserved (A) and newly synthesised (B) proteins for extracts of 12C. Values to the left refer to molecular weight markers, 14.3 – 220 kD, and values to the right of the gels represent approximate molecular weights of highlighted bands. Refer to Figure 3.3 B legend for description of lanes 1 - 10 in both gels.

A core group of proteins which is consistently expressed throughout the various temperature regimes imposed on 12C cultures are spread throughout from high to low molecular weight range (prefixed with * in Table 3.1.6, illustrated in Figure 3.5 A). These proteins are also close in molecular weight to a number of hsps, which have been identified in other eukaryotic systems. These include: 60.6 (hsp60), 30 (hsp30), 27.7 (hsp28), 26.4 (hsp26), 23.4 (hsp23), 22.4 (hsp22) and 12.1 kD (hsp12).

The range of newly synthesised proteins compared to those found in Bb24 extracts is far less, with only 10 recognisable proteins seen (Table 3.1.7). The range, unlike that for Bb24, which is concentrated between mid to high molecular weight regions, is expressed throughout the high to low sectors of the autoradiogram. In comparison to the continuously expressed proteins, none of the new proteins appear to correlate with known hsps.

Quantitative changes in relation to shifts in temperature were determined by studying the % IOD distribution throughout the lanes for any one particular protein. Table 3.1.8 depicts changes in % IOD for constitutively expressed proteins of interest in 12C extracts. Overall there is no fixed trend, which can be distinguished between all the proteins highlighted, but a complex pattern of synthesis occurs to each individual protein. The changes in the level of synthesis can be related to the various temperature regimes imposed. Increased and decreased synthesis for proteins expressed in cultures subjected to sublethal temperatures, Train A and B extracts (lanes 2 and 5 respectively) are compared to the control represented in lane 8 which are illustrated in Table 3.1.4 and prefixed with $^{+}$ or $^{-}$.

Molecular Weight	Lanes %IOD								
(kD)	2	3	4	5	6	7	8	9	10
*60.6	-2.33	8.46		-0.93	5.61		3.62		
*56.3	+7.57	5.49		-3.68	3.65		4.06	7.97	
*50	+6.55	7.29		-3.81	4.26		5.99		
*45	-4.96	4.54	52.92	+6.04	13.35		5.51	9.38	
33.7	-1.62	3.45	9.14	+3.67	2.55		2.79		
31.8	-1.74	5.93	15.40	+3.55	2.70		3.38		
*30	+12.00	11.22		-6.18	3.34		11.19	20.40	
*27.7	-3.28	6.19		+6.59	4.99		5.29	2.09	
*26.4	-2.85	6.79		+6.79	20.27		6.50	25.28	
*23.4	+9.67	3.41			4.59		2.23		
*20.3	+16.20	18.07	22.53	+7.03	3.01		4.01		
*15.2	+6.47	1.69		-0.89	1.41		1.43		
*10.5	-1.37	1.76		-1.85	3.01		2.10		

Table 3.1.8. Quantification of band intensities measured as %IOD of conserved proteins, which are expressed in extracts of 12C. Molecular weights prefixed with * relate to proteins illustrated in Figure 3.5 A. Values in lanes 2 and 5 prefixed with $^{+}$ or $^{-}$ correspond to increased or decreased synthesis respectively, in relation to control values in lane 8.

Decreased synthesis of 60.6 kD protein occurs in both Train A and B extracts, with approximately 1.5 and 4 fold decrease respectively, compared to its presence in the control extract. Upon exposure to extreme heat treatment, synthesis of this protein increases dramatically in both Train A (lane 3) and B extracts (lane 6) with % IOD's of 8.46 and 5.61 respectively. A similar trend occurs for 10.5 kD protein whereby there is decreased synthesis during sublethal temperature (Train A and B) conditions compared to the control, but upon exposure to extreme heat treatment there is increased synthesis.

Differences occur in the level of expression of proteins present in Train A and B extracts. For example, increased synthesis of a given protein in Train A may show a decrease in Train B when compared to the control counterpart. This is illustrated clearly for proteins 56.3, 50, 30,

and 15.2 kD, the reverse of this trend with increased synthesis in Train B and not Train A occurs for 45, 33.7, 31.8, 27.7 and 26.4 kD (Table 3.1.8).

The effect of extreme heat treatment (lanes 3, 6 and 9) upon trained and control cultures is also complex, increased synthesis occurs not only for 60.6 kD, previously mentioned but for 50, 26.4 and 10.5 kD proteins. Distinct differences in the levels of synthesis of 33.7, 31.8, 30, 27.7 and 20.3 kD proteins are also apparent when comparing the extreme heat treatment of Train A and B extracts. Increased synthesis occurs for Train A but not Train B extracts (lane 3 and 6), with the reverse of this trend occurring for only 15.2 and 45 kD.

A further layer of complexity can be observed for the levels of synthesis of 30 and 45 kD proteins present in control extreme heat treated extracts (lane 9). A 2 fold increase occurred when trained (A and B) samples were exposed to extreme temperatures.

Recovery of constitutively expressed proteins only appears for 4 proteins, which have been exposed to sublethal temperatures (Train A), 45, 33.7, 31.8 and 20.3 kD. It can be surmised that although the recovery profile of Train A does not resemble the control profile in lane 8, the presence of 4 proteins mentioned above support the possibility that if the cultures were left longer than 24 hr to recover, they may have resumed their original pattern of synthesis.

Proteins present only when cultures were exposed to a change in temperature and not in the control (lane 8) were also examined for any distinct changes in the level of synthesis with respect to the % IOD. Table 3.1.9 illustrates that overall there was an increase in the newly synthesised proteins for example, 29.5 (lane 5, band 13) and 48 kD (lane 9, band 4) with % IOD's of 7 and 8.28 respectively.

Molecular Weight	Lanes %IOD								
(kD)	2	3	4	5	6	7	8	9	10
66.7	1.21								
65.2	0.00			0.34					
61.8								8.28	
48					3.24				
41.9									
40	2.23			2.05					
29.5				7.00					
19.8				1.84	1.00				
14.4	0.52								

Table 3.1.9. Quantification of band intensities measured as %IOD of newly synthesised proteins, which are expressed in extracts of 12C, which have been exposed to a change in temperature.

Global band comparisons between the two species of *Serpula* were undertaken to establish the presence of conserved proteins common to both. Figure 3.6 A and B illustrates nine proteins ranging between 50.3 - 9.2 kD.

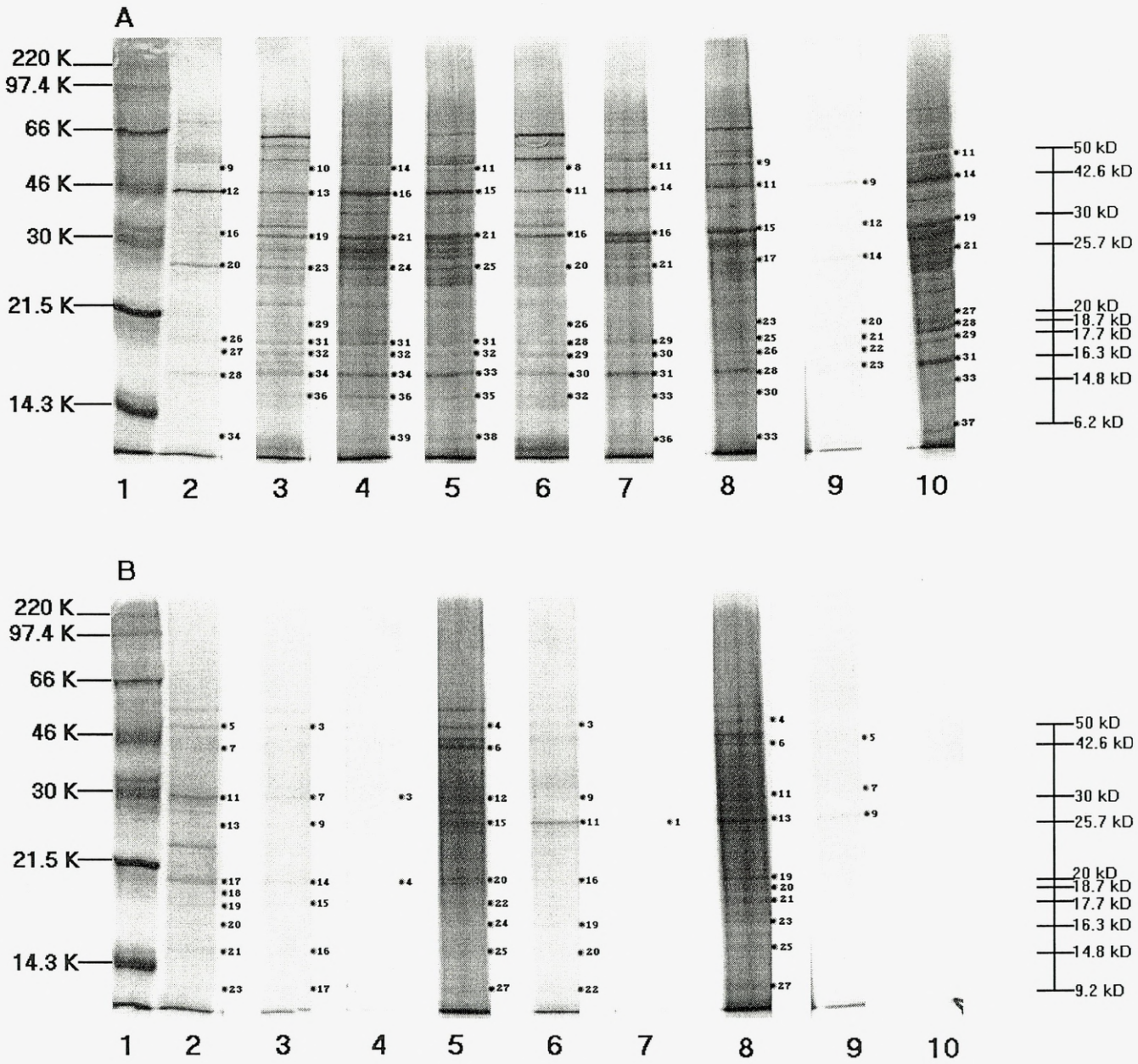


Figure 3.6 A and **B**. Comparative analysis of Bb24 and 12C autoradiograms depicting sets of proteins which are conserved in both species (molecular weights to the right of the gels). Refer to Figure 3.3 A and B legend for description of lanes 1 - 10 in both profiles.

Table 3.2.0 depicts the comparative study made between Bb24 and 12C to identify proteins which are constitutively synthesised in both *Serpula* species. Proteins with approximate molecular weights of 30, 26, 20, 19, 18 and 16 kD resemble in size hsp30, 26, 20, 19, 18 and 16 respectively.

Lanes																			
Bb24										12C									
Band (kD)	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Band (kD)**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
50.0	9	10	14	11	8	11	9		11	**50.4**	5	3		4	3		4		
42.6	12	13	16	15	11	14	11	9	14	**43.5**	7			6			6	5	
30.0	16	19	21	21	16	16	15	12	19	**30.0**	11	7	3	12	9		11	7	
25.7	20	23	24	25	20	21	17	14	21	**26.4**	13	9		15	11	1	13	9	
20.0		29			26		23	20	27	**20.3**	17	14	4	20	16		19		
18.7	26	31	31	31	28	29	25	21	28	**19.3**	18						20		
17.7	27	32	32	32	29	30	26	22	29	**18.5**	19	15		22			21		
16.3	29	34	34	33	30	31	28	23	31	**17.2**	20			24	19		23		
14.8		36	36	35	32	33	30		33	**15.2**	21	16		25	20		25		
9.2	34		39	38		36	33		37	**10.5**	23	17		27	22		27		

Table 3.2.0. Comparisons of a core set of proteins which are expressed in both Bb24 and 12C. Band numbers correspond to those indicated in Figure 3.3 A and B.

Overall, there are distinct similarities and differences between the two species. This can be summarised by the following points.

(i) Conserved proteins of high molecular weight were prevalent in Bb24 but not 12C, with the highest detectable protein in 12C of 60.6 kD, compared to Bb24 with 90 kD.

(ii) A similar protein in size to that of the AMPK protein p63, was detectable in extracts of Bb24 but not 12C.

(iii) Proteins similar in molecular weight hsp from the four major families of heat shock proteins were identifiable in both Bb24 and 12C, which are summarised in Table 3.2.1.

Hsp Family	Bb24 (kD)		12C (kD)	
	Constitutively	New	Constitutively	New
Hsp 90 (M_r 83 - 96 kD)	83.4,90	68.2,81.3, 94.8		
Hsp 70 (M_r 67 - 78 kD)	69.9,73.8	71.1		
Hsp 60/GroEL (M_r 58 - 65 kD)		59, 60.4	60.6	61.8,65.2
Small Hsps (M_r 12 - 44 kD)	12,14.3,16.3, 16.8,17.7,20, 20.6,21.7,23, 24.7,25.7,30, 34	13.5,20.8, 22.2,27.2, 32.4,	12.1,16.4, 17.2,17.8, 20.3,21,22.4, 23.4,25,26.4, 30	14.4,19.8, 26.9,29.5

Table 3.2.1. Summary of proteins expressed in Bb24 and 12C extracts, which are related by molecular weight to hsps.

3.1.4 Further Examination of Newly Synthesised Proteins by 2-D SDS PAGE.

Since O'Farrell (1975) introduced the technique for high-resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), it has become one of the most powerful tools for the separation and quantification of complex mixtures of proteins. This technique employs the separation of denatured proteins according to their molecular weight and isoelectric point, with sufficient resolution to allow individual proteins to appear as discrete spots on a polyacrylamide gel. Due to its resolution, the 2-D PAGE technique has been applied to vast number of biological systems (Cash, 1991; Tissot and Spertini, 1995).

Many environmental stresses result in alterations in the patterns of protein synthesis in prokaryotic and eukaryotic cells, and there is often overlap between the sets of polypeptides induced by various stresses. Studies illustrating the comparative analysis of stress factors such as UV radiation, oxygen, and ethanol shock upon *Rhodobacter sphaeroides* (Nepple and Bachofen, 1997) and salt stress, water deficit and osmotic stress in barley roots (Hurkman and Tanaka, 1988), are but two of the many examples in which 2-D PAGE was used.

The aims of the research outlined below were as follows.

(i) To apply 2D PAGE for the analysis and comparison of protein profiles of *S. cerevisiae* wine yeast, L-2226 and the two *Serpula* species, for the identification of any changes due to the temperature regimes imposed.

(ii) To show any specific changes due to changes in temperature which can be correlated by molecular weight to hsps.

Initially control experiments using non-labelled intracellular yeast extracts were run to test the 2-D NEPHGE system used. The following silver stained profiles represents the changes in total protein in relation to the various temperature regimes imposed on yeast, L-2226 cultures (Figure 3.7).

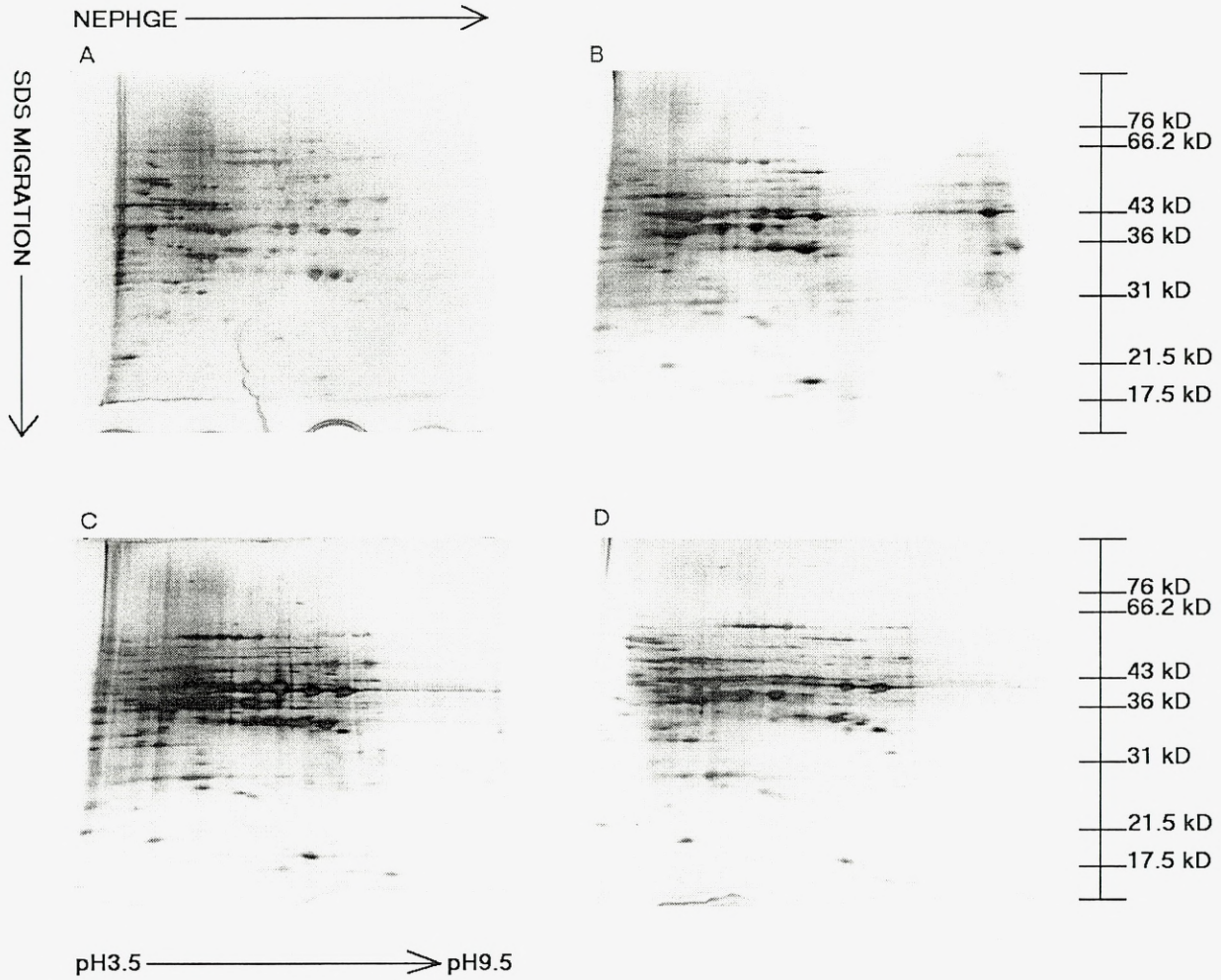


Figure 3.7 A - D. Two-dimensional silver stained profiles of the separation of total proteins synthesised by yeast (wine strain L-2226) cultures, which have under gone sequential temperature regimes. (A) Control cultures grown at optimum conditions of 30 ^{0}C /1 hr. (B) Subjected to a sublethal temperature of 42 ^{0}C /1 hr. (C) Exposure to extreme heat of 52 ^{0}C /1 hr. (D) Placed into recovery at 30 ^{0}C /1 hr. All 1-dimensional loading were equalised for approximately 6 - 10 μg. Values to the right of the gels represents the location of molecular weight standards analysed on parallel marker gels, ranging from 17.5 - 76 kD (Bio-Rad 2-D SDS-PAGE standards, cat. 161-0320).

2-D autoradiograms of Bb24 intracellular extracts of cultures grown at the various temperature regimes outlined in Chapter 2, section 2.1.1.2, are represented in Figure 3.8. The resolution of proteins separated for 12C intracellular extracts was not as clearly defined as compared to those of Bb24 extracts, resulting in incomplete analysis of the autoradiograms (data not shown).

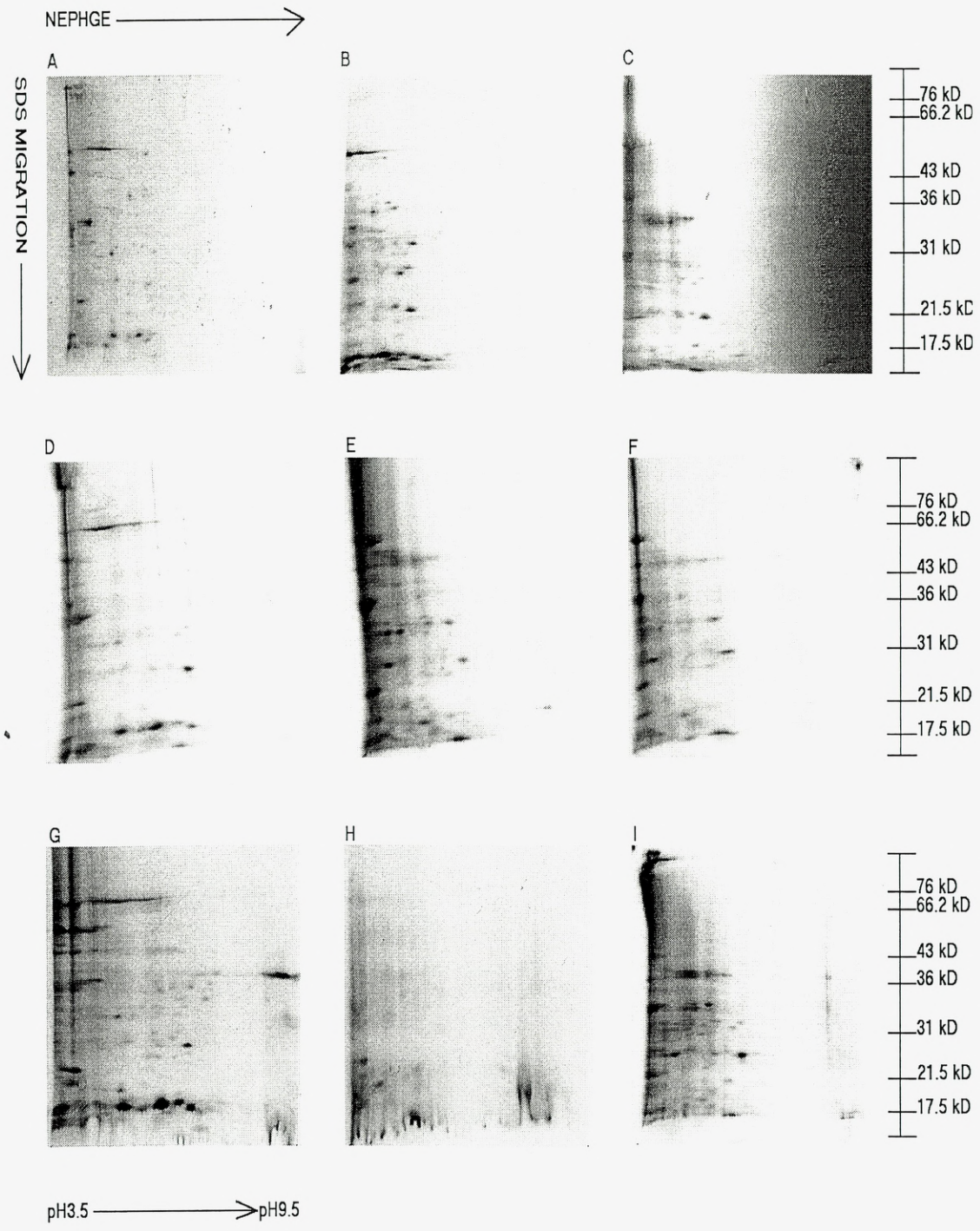


Figure 3.8 A - I. Two-dimensional autoradiogram profile of Bb24 Train A, B and control (Train C) cultures. Each pattern corresponds to one of the synthesised polypeptide profiles observed by investigating the different stages of the temperature regimes imposed.

Figure 3.8 A - C. Train A cultures: (A) cultures first exposed to sublethal temperature of 28 ^{0}C for 48 hr; (B) then exposed to extreme heat treatment of 40 ^{0}C for 3 hr; (C) after which they are placed into recovery after the extreme treatment at optimum conditions of 25 ^{0}C for 24 hr.

D - F, Train B cultures: (D) cultures first exposed to sublethal temperature of 30 ^{0}C for 48 hr; (E) cultures then exposed to extreme heat treatment of 40 ^{0}C for 3 hr; (F) placed into recovery after extreme treatment at optimum conditions of 25 ^{0}C for 24 hr.

G - I, Train C (Control) cultures: (G) cultures grown at optimum conditions of 25 ^{0}C for 24 hr; (H) cultures exposed extreme heat treatment of 40 ^{0}C for 3 hr and (I) placed into recovery after extreme treatment at optimum conditions of 25 ^{0}C for 24 hr.

All 1-dimensional loading were equalised for approximately 60 - 80 μg. Values to the right of the gels represents the location of molecular weight standards analysed on parallel marker gels, ranging from 17.5 - 76 kD (Bio-Rad 2-D SDS-PAGE standards, cat. 161-0320).

The data presented above were obtained using NEPHGE in the first dimension of 2-D SDS PAGE. Under these conditions described previously in section 2.3, approximately 100 - 150 proteins in total were resolved for L-2226 yeast extracts (Figure 3.7) and 30 - 60 for Bb24 extracts (Figure 3.8).

For computer-assisted gel matching, images (1024 x 1024 pixels) of stained and autoradiographed gels were captured as described in Section 2.4.4. and transferred to "Phoretix" 2-D Lite analytical software version 3.51 for initial analysis and transferred to Bio Image Intelligent Quantifier for full analysis. The protein spots were detected by a combination of automatic spot detection protocols, and manual editing used to calculate the p*I*'s and molecular weights.

The data presented in Figures 3.7 and 3.8 demonstrated that a proportion of the proteins resolved by 2-D PAGE were constitutively expressed in all the temperature regimes imposed for both *S. cerevisiae* (L-2226) and *S. himantioides* (Bb24). It is also clearly shown that there are a number of proteins, which are expressed at increased temperatures only.

Figures 3.9 and 3.10 A - D show core sets of constitutively and newly synthesised proteins present in the intracellular L-2226 extracts, with estimated values for p*I* and M_r summarised in Table 3.2.2 and 3.2.3 respectively.

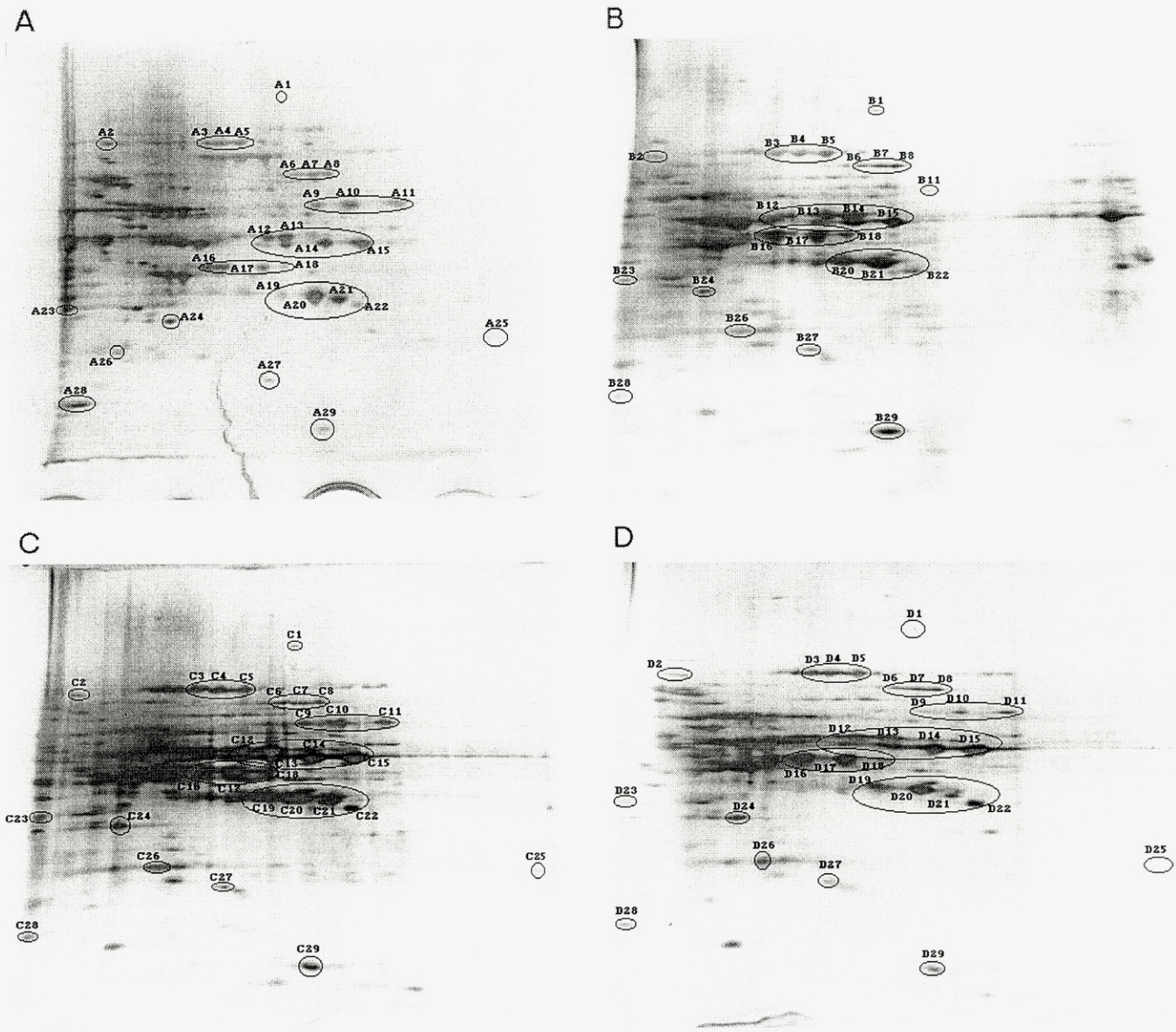


Figure 3.9 A - D. Comparative analysis of L-2226 protein profiles depicting sets of proteins, which are constitutively present in all treatment regimes used. (A) Represents the control, cultures grown at 30 ^{0}C for 1 hr, (B) cultures are then subjected to a sublethal temperature of 42 ^{0}C for 1 hr, after which, (C) exposed to extreme heat treatment of 52 ^{0}C for 1 hr and finally placed into recovery (D) back into optimal conditions of 30 ^{0}C for 1 hr. Highlighted regions with labelled proteins correspond to values presented in Table 3.2.2.

Table 3.2.2 summarises core sets of proteins i.e. constitutively present, in all the treatment regimes imposed on L-2226 cultures illustrated in Figure 3.9.

Spot number in reference profiles in Figure 3.9	Spot number in master profiles Figure: A(Opt)	B(Tr)	C(HS)	D(R)	Estimated p*I*	Estimated M_r ($x10^3$)
1	102	57	4	93	5.6	67.8
2	2	7	88	96	4.1	54.2
3	126	4	6	2	4.9	53.4
4	4	5	10	10	5.1	53.1
5	3	6	11	3	5.3	53.2
6	154	8	206	80	5.5	52.4
7	10	9	117	5	5.6	51.5
8	11	10	15	6	5.7	50.2
9	18		23	131	5.6	46.6
10	63		222	11	5.7	47.5
11	62	65	24	76	5.9	44.8
12	29	97	155	120	4.9	41.7
13	27	85	135	119	5.2	41.2
14	34	34	117	121	5.6	40.7
15	36	32	128	122	5.6	40.3
16	40	33	248	110	4.9	39.1
17	43	35	120	109	5.1	39.0
18	91	95	45	40	5.4	38.2
19	90		200	124	5.3	36.0
20	52	46	201	123	5.6	35.7
21	170	47	245	139	5.6	35.6
22	64	49	89	61	5.7	34.7
23	58	83	63	90	3.6	31.0
24	60	51	65	135	4.3	30.7
25	132		97	89	8.8	27.9
26	115	54	70	68	4.5	26.7
27	80	105	78	72	4.9	25.5
28	61	76	74	84	3.3	23.1
29	79	56	76	70	5.6	19.9

Table 3.2.2. Summary of protein matching between core sets of proteins present in all silver stained L-2226 profiles with estimated p*I* and molecular weights (M_r). Comparisons made against master gel profile, which is represented by the L-2226 control profile shown in Figure 3.9A.

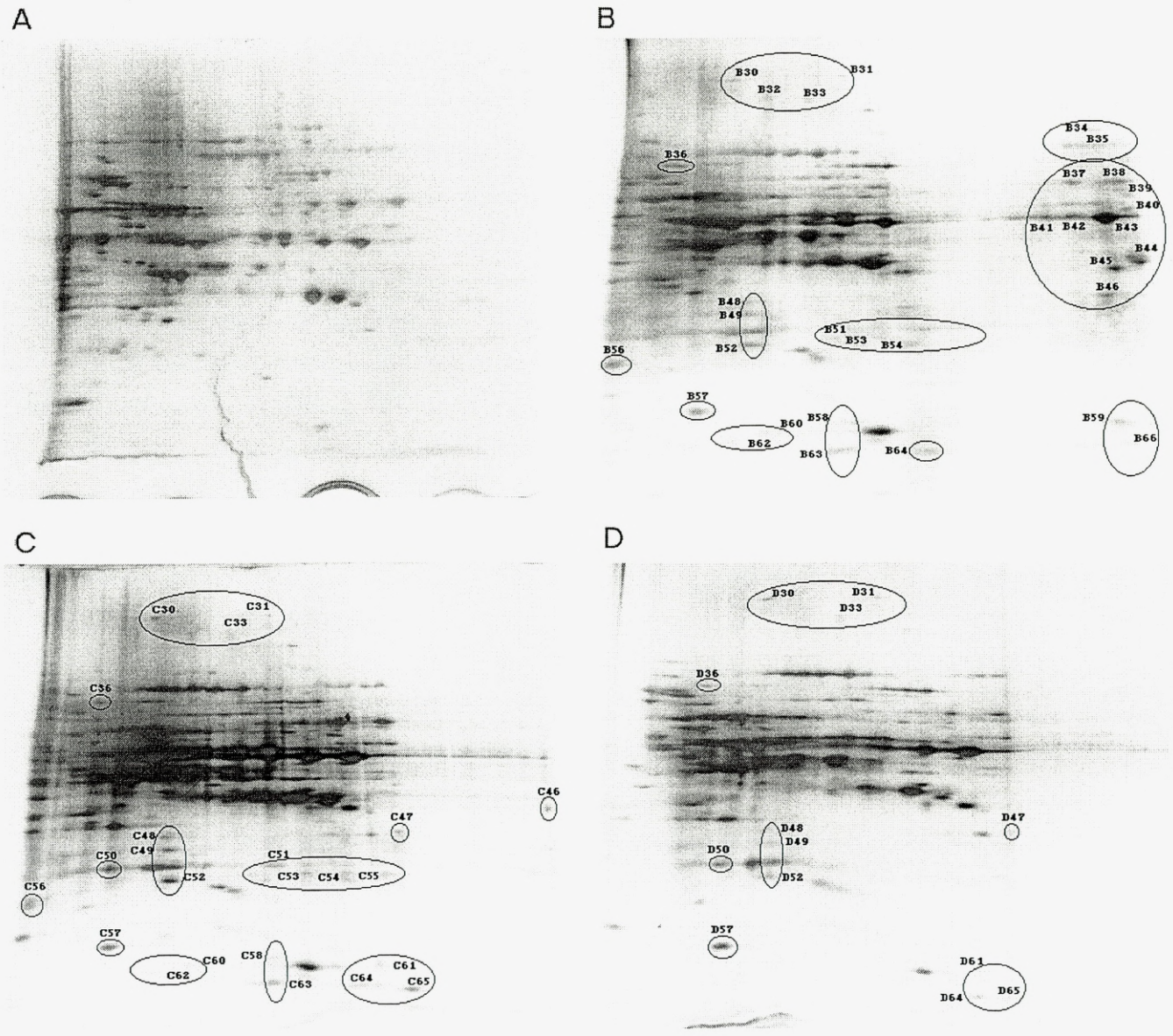


Figure 3.10 A - D. Comparative analysis of L-2226 protein profiles depicting sets of proteins, which are newly synthesised due to shift in temperature. (A) Represents the control, cultures grown at 30 ^{0}C for 1 hr, (B) cultures are then subjected to a sublethal temperature of 42 ^{0}C for 1 hr, after which, (C) exposed to extreme heat treatment of 52 ^{0}C for 1 hr and finally placed into recovery (D) back into optimal conditions of 30 ^{0}C for 1 hr. Highlighted regions with labelled proteins correspond to values presented in Table 3.2.3.

Table 3.2.3 summarises the sets of proteins, which were newly synthesised due to a change in temperature imposed on L-2226 cultures illustrated in Figure 3.10.

Spot number in reference profiles in Figure 3.10	Spot number in master profiles Figure: A(Opt)	B(Tr)	C(HS)	D(R)	Estimated pI	Estimated M_r ($x10^3$)
30		61	3	91	4.6	77.9
31		58	79	92	5.5	79.9
32		115			4.9	71.9
33		59	2	146	5.1	72.5
34		67			8.6	65.3
35		159			8.2	62.5
36		80	188	97	4.3	49.0
37		66			8.0	48.9
38		12			8.7	43.7
39		119			8.8	42.6
40		107			8.6	40.8
41		27			7.5	40.0
42		28			7.8	40.0
43		31			8.3	39.8
44		41			8.7	36.2
45		48			8.5	34.3
46		52	93		8.4	30.7
47			90	75	6.5	31.3
48		101	95	144	4.6	30.2
49		103	68	87	4.7	38.7
50			71	67	4.2	27.3
51		129	91		5.4	27.7
52		104	72	86	4.6	26.5
53		124	92		5.6	27.1
54		109	243		5.9	27.4
55			109		6.5	27.6
56		75	100		3.4	25.2
57		74	75	69	4.2	19.9
58		116	212		5.4	20.5
59		68			8.8	17.3
60		73	211		4.8	20.7
61			105	145	6.6	19.8
62		72	246		4.5	18.2
63		70	98		5.3	18.4
64		71	103	73	6.6	18.4
65			77	74	7.7	18.3
66		69			9.0	15.4

Table 3.2.3. Summary of protein matching between newly synthesised proteins present in all silver stained L-2226 profiles with estimated p*I* and molecular weights (M_r).

The 2-D analysis of total protein content present in intracellular extracts of L-2226 cultures, which have been exposed to heat stress, illustrates distinct changes. A number of proteins were identified as being present throughout the temperature regimes applied and these are clearly illustrated in Figure 3.9. Estimated values corresponding to the p*I* and M_r for the highlighted proteins are summarised in Table 3.2.2 show a wide range of molecular weights from 67.8 kD to 19.9 kD. There are approximately 6 subsets of proteins (A/B/C/D3 - 22; Figure 3.9) which are clearly identified in all the extracts analysed and appear to be a core set of proteins synthesised. These proteins range between 54 - 34 kD with p*I*'s of 4.9 -5.7. One subset, proteins 9 to 11 (corresponding to 46.6, 47.5 and 44.8 kD respectively) appear to diminish in intensity when cultures were exposed to sublethal temperature of 42 ^{0}C/ 1 hr (Figure 3.9 B), but increases when cultures were subjected to 52 ^{0}C / 1hr (Figure 3.9 C). When cultures are placed back into optimal conditions of 30 ^{0}C / 1 hr, the intensity once again appears to decrease. A similar trend can be seen for protein 23 (31 kD, *pI* of 3.6, referred to as A/B/C/D23 in Figure 3.9).

There are groups of proteins that appear to show a linear train of spots with similar M_r but with different p*I*'s. The formation of these proteins in a an extended train may represent heterogeneity of the proteins possibly resulting from post-translational modifications *in vivo*, or the products of a multigene family. Alternatively, the line of polypeptides could be a result of artefacts of sample preparation and electrophoresis, but the consistency of the results would argue against this.

When comparing the 2-D profiles for changes in the protein patterns as a result of temperature shifts, there is a prominent group of basic proteins which appear to be expressed only during the sublethal temperature regime, 42 ^{0}C / 1hr (groups B34 - 35 and B37 - 46 depicted in Figure 3.10 B). These proteins lie within the 65.3 - 30 kD molecular weight range. Within the

B37 - 46 group there is a subset B41 - 43 that have molecular weights of approximately 40 kD with slightly different p*I*'s (Refer to Table 3.2.2 for individual values). The last protein of this subset, B43 protein (38.9 kD with p*I* of approximately 8.3, Table 3.2.2) has the greatest intensity. The only protein out of this basic group (B37 - 46) which appears to be expressed during the subsequent temperature regime was B46 (assigned C46 in Figure 3.10 C) with an estimated molecular weight of 30.7 kD, p*I* of approximately 8.4.

A group of high molecular weight proteins (ranging from 77.9 - 72.5 kD within the mid *pI* of approximately 4.6 - 5.5, refer to Table 3.2.3 for individual values) appeared during the sublethal, extreme heat and recovery regimes as illustrated in Figure 3.10 B - D. Within this high molecular weight group, protein 32 shows a different pattern of expression being absent from the recovery phase (Figure 3.10 D). In addition, there are a number of small molecular weight proteins ranging from approximately 27 - 15 kD which are expressed as a result of heat stress (Table 3.2.3).

An intriguing observation is that a number of the proteins, which are present only during elevated temperatures, correspond in molecular weight range that is characteristic of important hsps (Table 3.2.3). Proteins 30 - 33 fall within the hsp70 family (M_r 67 - 78 kD) while proteins 33 and 34 which are only expressed during the 42 ^{0}C incubation fall in the molecular range of the hsp60/GroEL family (M_r 58 - 65 kD). The whole range of small molecular weight proteins encompasses the molecular weights of the small hsp family (M_r 12 - 44 kD). In addition, proteins present in both 42 ^{0}C and 52 ^{0}C and corresponding in M_r to known hsps are the follows: 30.7 kD (hsp30); 27.7 kD (hsp28); 25.2 kD (hsp25) and 20.7 kD (hsp21). Two subsets of small molecular weight proteins with similar size but slightly different p*I*'s are group 51, 53, 54 having a molecular weight similar in size to hsp27 and group 62, 63, 64, 65 corresponding in size to hsp18 (refer to Table 3.2.3).

The data presented suggests that the heat-shock response can be evaluated using the 2D SDS-PAGE system. The next stage was the analysis of Bb24 autoradiograms to evaluate any changes in protein synthesis in relation to the various temperature regimes applied. Figure 3.11 A - I illustrates the presence of core sets of constitutively and newly synthesised proteins expressed in Bb24 intracellular extracts. Proteins highlighted by circles and rectangles refer respectively to constitutively expressed and newly synthesised proteins.

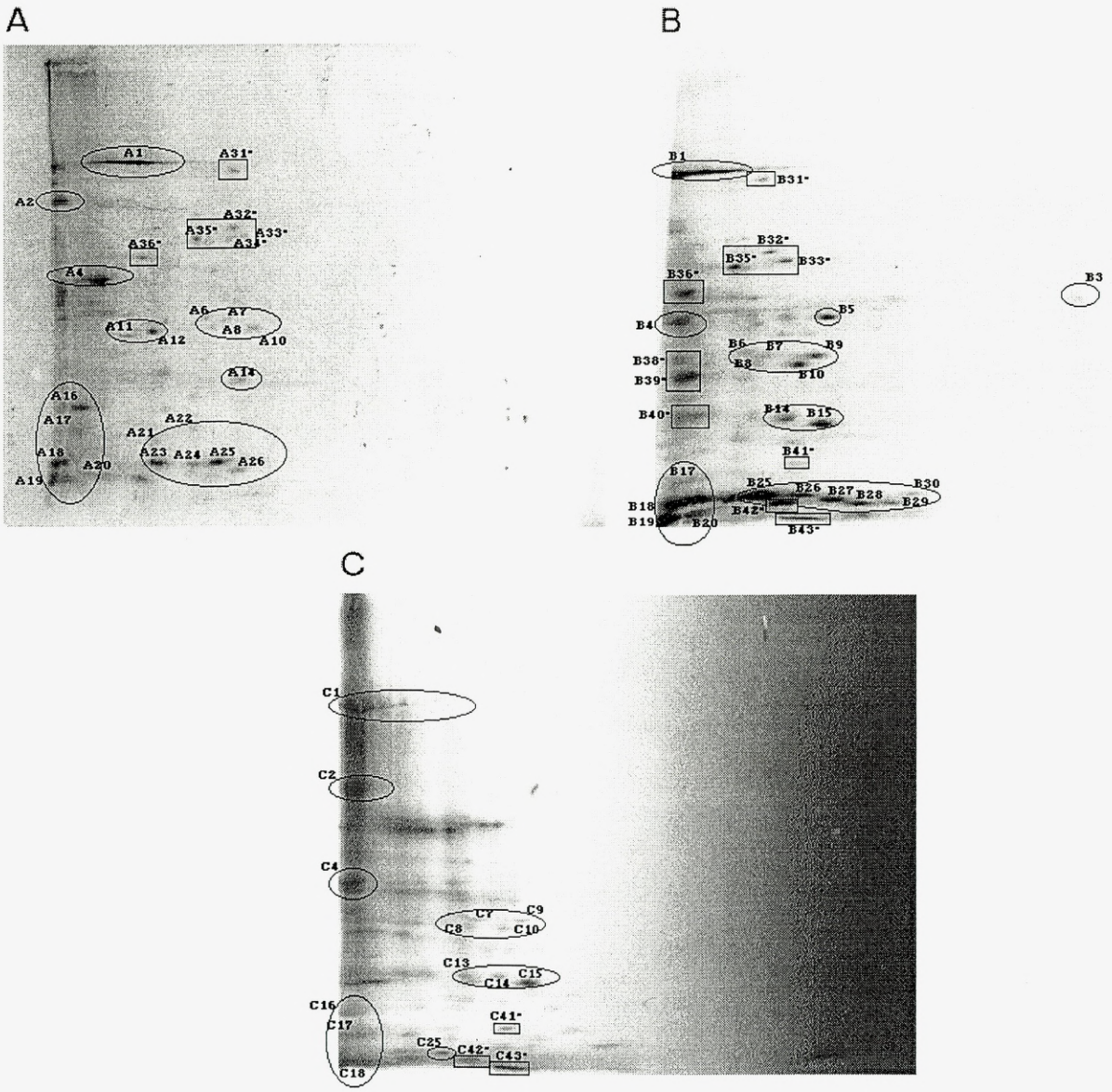


Figure 3.11 A - C. Comparative analysis of Bb24 Train protein profiles depicting sets of proteins, which are constitutively and newly synthesised (highlighted by circle or rectangle, respectively). (A) Represents cultures preconditioned to Train A temperature regime (28 ^{0}C/ 48 hr). (B) Represents Train B cultures exposed to extreme heat treatment (40 ^{0}C/ 3 hr). (C) Represents extreme heat-treated cultures placed back into recovery conditions (25 ^{0}C/ 24 hr).

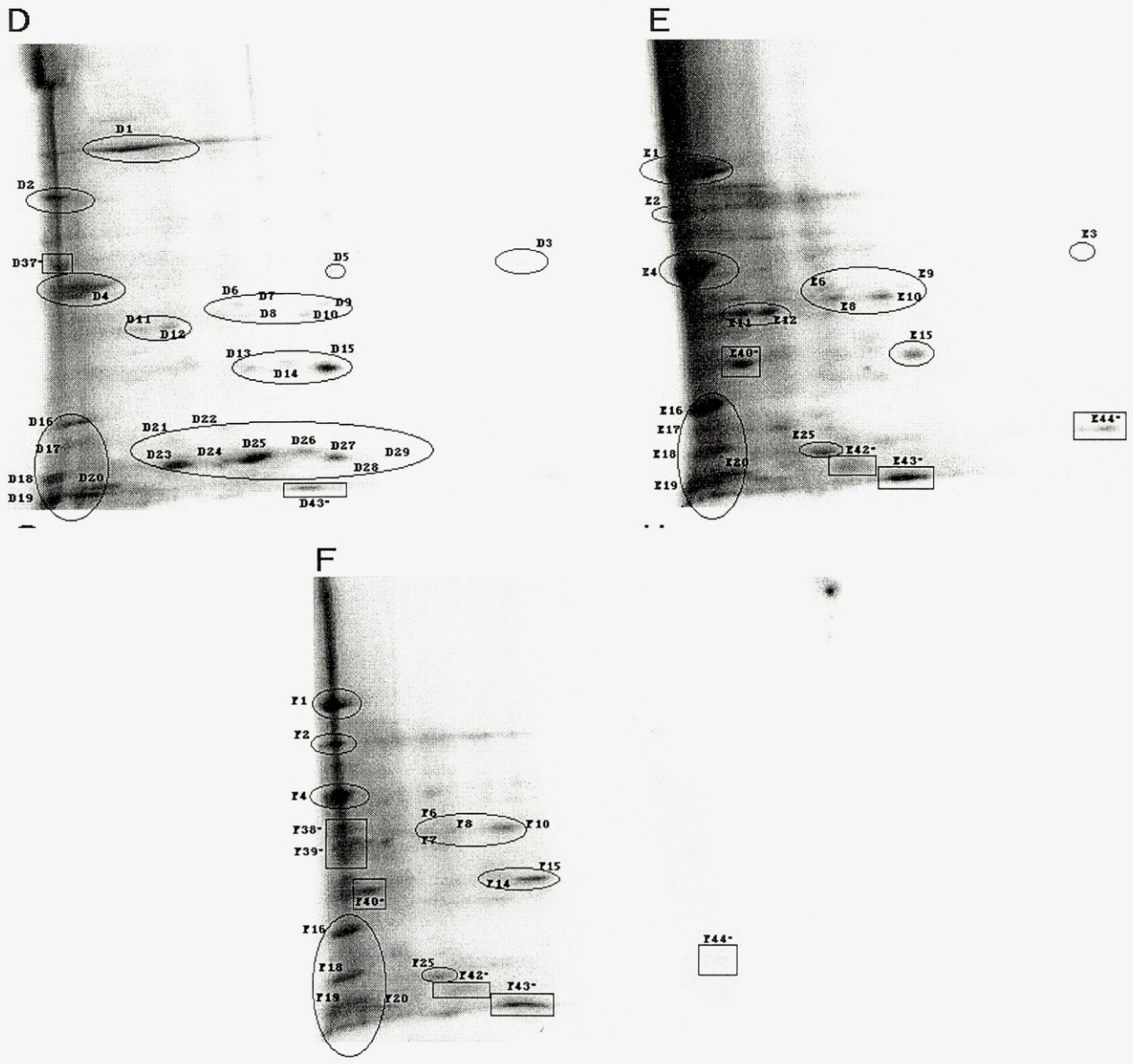


Figure 3.11 D - F. Comparative analysis of Bb24 Train B protein profiles depicting sets of proteins, which are constitutively and newly synthesised (highlighted by circle or rectangle, respectively). (D) Represents cultures preconditioned to Train B temperature regime (30 ^{0}C/ 48 hr). (E) Represents Train B cultures exposed to extreme heat treatment (40 ^{0}C/ 3 hr). (F) Represents extreme heat-treated cultures placed back into recovery conditions (25 ^{0}C/ 24 hr).

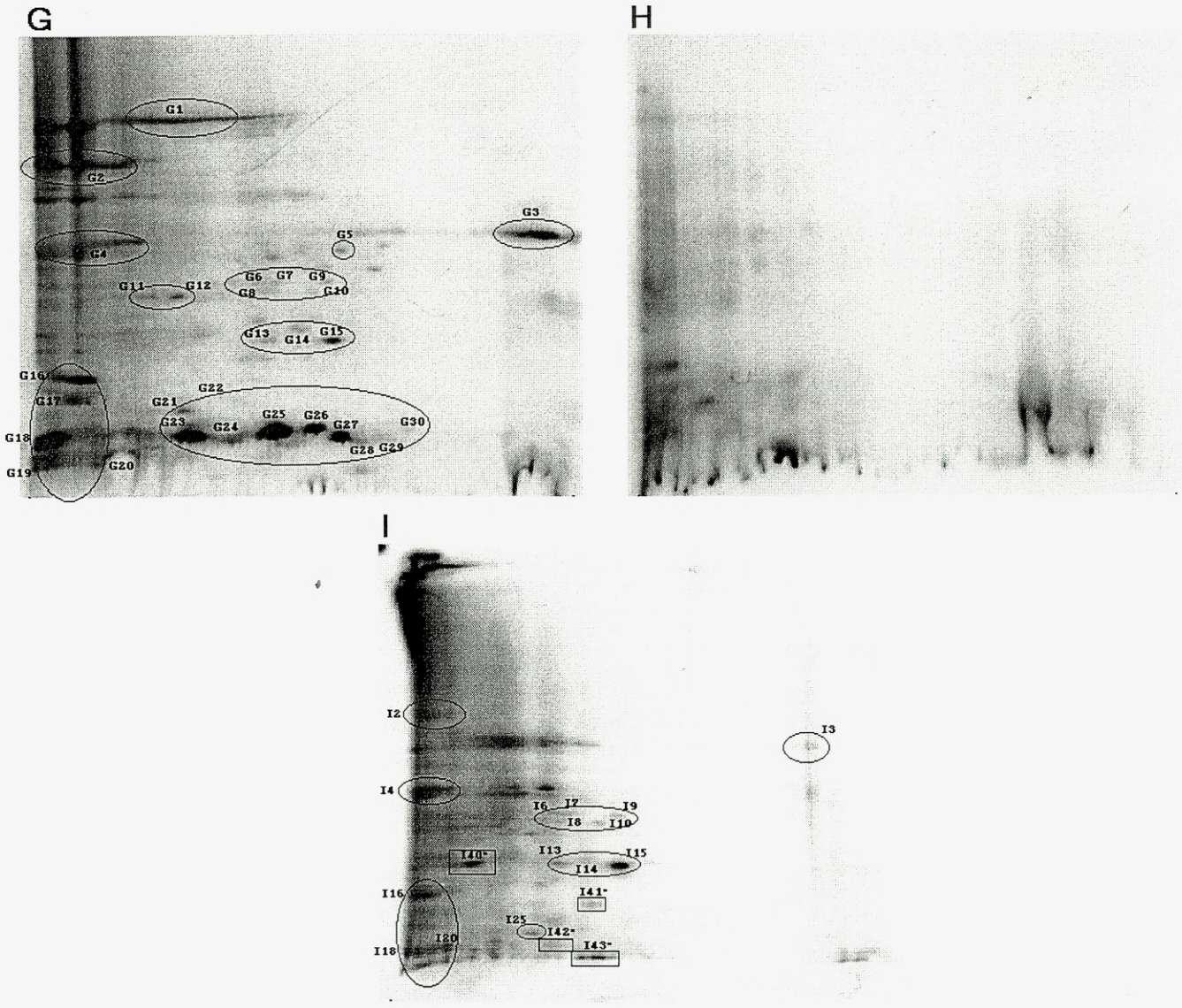


Figure 3.11 G - I. Comparative analysis of Bb24 Train C protein profiles depicting sets of proteins, which are constitutively and newly synthesised (highlighted by circle or rectangle, respectively). (G) Represents control cultures, Train C grown at optimum conditions (25 ^{0}C/ 24 hr). (H) Represents Train C cultures exposed to extreme heat treatment (40 ^{0}C/ 3 hr). (I) Represents extreme heat-treated cultures placed back into recovery conditions (25 ^{0}C/ 24 hr).

Table 3.2.4 summarises the core sets of constitutively expressed proteins (highlighted by circles illustrated in Figure 3.11) present in Bb24 intracellular extracts.

Spot number in reference profiles represented in Figure 3.11	Spot number in master profiles									Estimated pI	Estimated M_r ($x10^3$)
	A	B	C	D	E	F	G	H	I		
1	56	1	93	4	41	2	38			4.7	77.3
2	3		15	6	44	4	6		46	4.1	68.0
3		25		39	20		5		27	7.4	44.6
4	7	57	95	38	43	32	57		8	4.1	45.3
5		26		40			9			5.7	41.1
6	31	42		30	2	28	61		35	4.9	35.0
7	32	44	66	32		27	62		36	5.0	34.6
8	58	43	84	31	4	29	63		42	4.9	33.1
9		6	21	34	18		44		22	5.4	33.8
10	20	7	22	35	3	7	45		23	5.3	31.0
11	30			50	5		70			4.3	34.5
12	10			26	6		11			4.4	34.6
13			85	36			32		31	4.8	27.4
14	19	11	27	35		40	48		30	5.0	27.8
15		12	10	12	7	9	13		11	5.5	26.9
16	21		12	14	50	11	55		14	4.1	24.5
17	29	49	39	13	52		54			4.1	22.3
18	12	68	48	20	49	21	29		15	3.9	19.4
19	16	67		21	47	24	24			3.8	15.6
20	28	69		22	48	25	30		4	3.9	18.3
21	27			27			15			4.3	22.1
22	26			28			73			4.5	22.7
23	13			19			18			4.3	19.6
24	25			16			75			4.5	20.6
25	14	61	13	24	12	12	28		26	4.6	19.9
26	22	13		25			27			5.3	20.2
27		18		13			21			5.6	20.2
28		19		10			20			5.8	19.7
29		47		11			51			6.0	20.8
30		48					52			6.2	21.9

Table 3.2.4. Summary of protein matching between core sets of conserved proteins present in Bb24 autoradiographic profiles with estimated p*I* and molecular weights (M_r). Comparisons were made against master gel profile of Bb24 control (Train C) represented in Figure 3.8 G.

Table 3.2.5 summarises the newly synthesised proteins (highlighted by rectangles, illustrated in Figure 3.11) present in Bb24 intracellular extracts which were expressed upon a shift in temperature.

Spot number in reference profiles represented in Figure 3.11	Spot number in master profiles									Estimated *pI*	Estimated M_r ($x10^3$)
	A	B	C	D	E	F	G	H	I		
31*	17	88								5.0	76.9
32*	18	2								4.9	65.5
33*	35	3								5.1	62.7
34*	36									5.0	61.2
35*	4	4								4.6	60.0
36*	42	82								4.2	53.3
37*										4.1	49.5
38*		39				33				4.0	38.5
39*		8				34				4.1	31.1
40*		93			8	10			10	4.1	27.9
41*		27	28						29	5.3	24.3
42*		62	46		21	18			25	5.0	18.7
43*		65	45	23	15	42			24	5.2	17.7
44*					19	20				7.5	25.8

Table 3.2.5. Summary of protein matching between newly synthesised proteins present in Bb24 autoradiographic profiles with estimated p*I* and M_r. Comparisons made against master gel profiles which are represented in Appendix J, J8. Numbers depicted with '*' refer to newly synthesised proteins annotated in Figure 3.11.

Analysis of the autoradiograms was not as straightforward as the 2-D profiles for yeast extracts mainly due to poor resolution and solubility of proteins. The range and number of proteins was far less with approximately 30 - 60 proteins resolved compared to that of the yeast profiles. The number of newly synthesised proteins resulting from exposure to elevated temperatures was also comparatively small with the highest number present in Train A culture exposed to 40 ^{0}C (Figure 3.11 B, new proteins highlighted by rectangles) with 11.

As with yeast there are groups of constitutive proteins present in all the extracts analysed with the exception of Train C extreme heat-treated cultures (Figure 3.11 H) where there are no detectable proteins. The overall pattern of synthesis illustrated in Figure 3.11 corresponds to the pattern depicted in the 1-D autoradiographic profiles (Figure 3.1), with no detectable synthesis of proteins occurring at 40 ^{0}C for control cultures (Figure 3.1, lane 9 and Figure 3.11H). As illustrated in Figure 3.11 the constitutively expressed proteins range from high to low molecular weights.

Two prominent high molecular weight proteins located at the acidic end which are expressed in most of the temperature regimes are proteins 1 and 2 (M_r approximately 77.3 kD, p*I* of 4.7 and 68 kD, p*I* of 4.1, respectively, Table 3.2.4). At the basic end the only protein expressed (M_r approximately 44.6 kD, p*I* of 7.4, Table 3.2.4), which is present in most of the temperature regimes is protein 3 (depicted as B/D/E/G/I3 in Figure 3.11 B, D, E, G and I).

Two groups within the mid range which appear to be always present are expressed proteins 6 - 10 and 13 - 15 (M_r range of approximately 31 - 33 kD and 26.9 - 28.7 kD respectively, with estimated p*I*'s of 4.8 - 5.5, refer to Table 3.2.4 for individual values). There are a group of low molecular weight proteins (group 16 - 20) also expressed throughout the various treatments at the acidic end (M_r of approximately 18.3 - 24.5 kD with estimated p*I*'s of 3.8 - 4.1, refer to Table 3.2.4). There is a linear train of low molecular proteins (group 23 - 29) which have similar M_r but different p*I*'s (M_r approximately 20 kD with estimated p*I* range between 4.3 - 6, refer to Table 3.2.4 for individual values). The complete group are expressed only in the control, Train C and sublethal regime, Train B cultures (Figure 3.11 G and D respectively) with some members of the group undetectable in the other temperature regimes. This is clearly illustrated in all the recovery extracts with protein 25 (depicted as C/F/I25 in Figure 3.11C, F and I) only expressed.

As was mentioned earlier the numbers of proteins expressed due to a shift in temperature are relatively small in comparison to yeast extracts. There are two groups of proteins which appear only to be expressed in Bb24 cultures incubated at sublethal temperature of 28 ^{0}C (Train A) and subsequent exposure to 40 ^{0}C. Protein 31* (depicted as A/B31* in Figure 3.11 A and B) is similar in molecular weight to (approximately 76.9 kD with estimated p*I* of 5.0) the hsp70 family. A cluster of 4 proteins (proteins 32 - 35*) fall within the same M_r range of the hsp60/GroEL family, having M_r of approximately within 60 - 65 kD, with estimated p*I*'s of 4.6 - 5.1 (refer to Table 3.2.5 for individual values). Proteins, which have similar Mr to known sHSPs, are depicted in Table 3.2.5 as the following: 27.9 kD (hsp28); 25.8 kD (hsp26) and 17.7 kD (hsp18).

In summary it has been clearly demonstrated the fine resolution obtained for L-2226 yeast allowed analysis of heat-treated cultures to be studied. This organism has been shown to have a remarkable property of yielding excellent separation patterns over a wide range of electrophoresis conditions (Brousse et. al., 1985; Bataillé et. al., 1987, 1988, 1991; Norbeck and Blomberg, 1997). The profiles for yeast also illustrates the power of 2-D SDS PAGE with respect to identifying specific changes in protein profiles resulting from exposure to heat stress. This was illustrated for a group of basic proteins which were present only during the sublethal temperature regime, 42 ^{0}C/ 1 hr (Figure 3.10 B) which upon exposure to a higher temperature regime the proteins disappeared. By contrast the resolution of proteins for Bb24 extracts were not as clear, but it was still possible to show changes in protein synthesis with respect to shifts in temperature. Proteins relating in molecular weight to hsps could also be identified.

3.15 *In Vitro* Translation Studies: The Analysis of Changes in mRNA Expression.

In most eukaryotes, cells respond to mild heat treatments by inducing heat-shock genes. Numerous studies have shown that within minutes of temperature elevation in for example, *Drosophila* cells, hsp genes vigorously transcribe mRNA's for heat-shock proteins (Lindquist, 1980; Scott and Pardue, 1981).

Data from the SDS-PAGE analysis indicate that that many putative hsps may well be induced in *Serpula* species. To further analyse this response, the translational analysis of poly (A^+) tail mRNA was initiated. The data shown in Figure 3.12 A and B illustrate the synthesis of a number of proteins from Bb24 and 12C mRNA extracts, using the rabbit reticulocyte lysate system. Full analysis of the molecular weights and Rf values are displayed in the Appendix J, J9 and 10.

It is seen that the mRNAs express 6 proteins, which are present in both extracts of Bb24 and 12C, although they are poorly translated. The only difference occurring with an absence of bands in the extreme temperature and recovery profiles for 12C (lane 9 and 10, Figure 3.12 B), suggesting that mRNA synthesis was reduced or absent. This is consistent with the observation that control cultures are no longer viable after extreme heat treatment of 40 ^{0}C for 2 hr. The extreme heat profile for 12C controls (lane 9, Figure 3.12 B) apparently displays 2 bands, running at approximately 70 and 50 kD. Expression occurs throughout the temperature regimes applied for Bb24 extracts. The proteins approximately run at 90, 69, 60, 50, 30 and 14.5 kD. A core set which are constitutively expressed in both species correlate to those seen in the intracellular profiles presented in Figure 3.6 A and B, namely 50, 30 and 14.5 kD. The proteins 90, 69 and 60 kD fall into the M_r range of three hsp families, hsp90, hsp60/GroEL and hsp70.

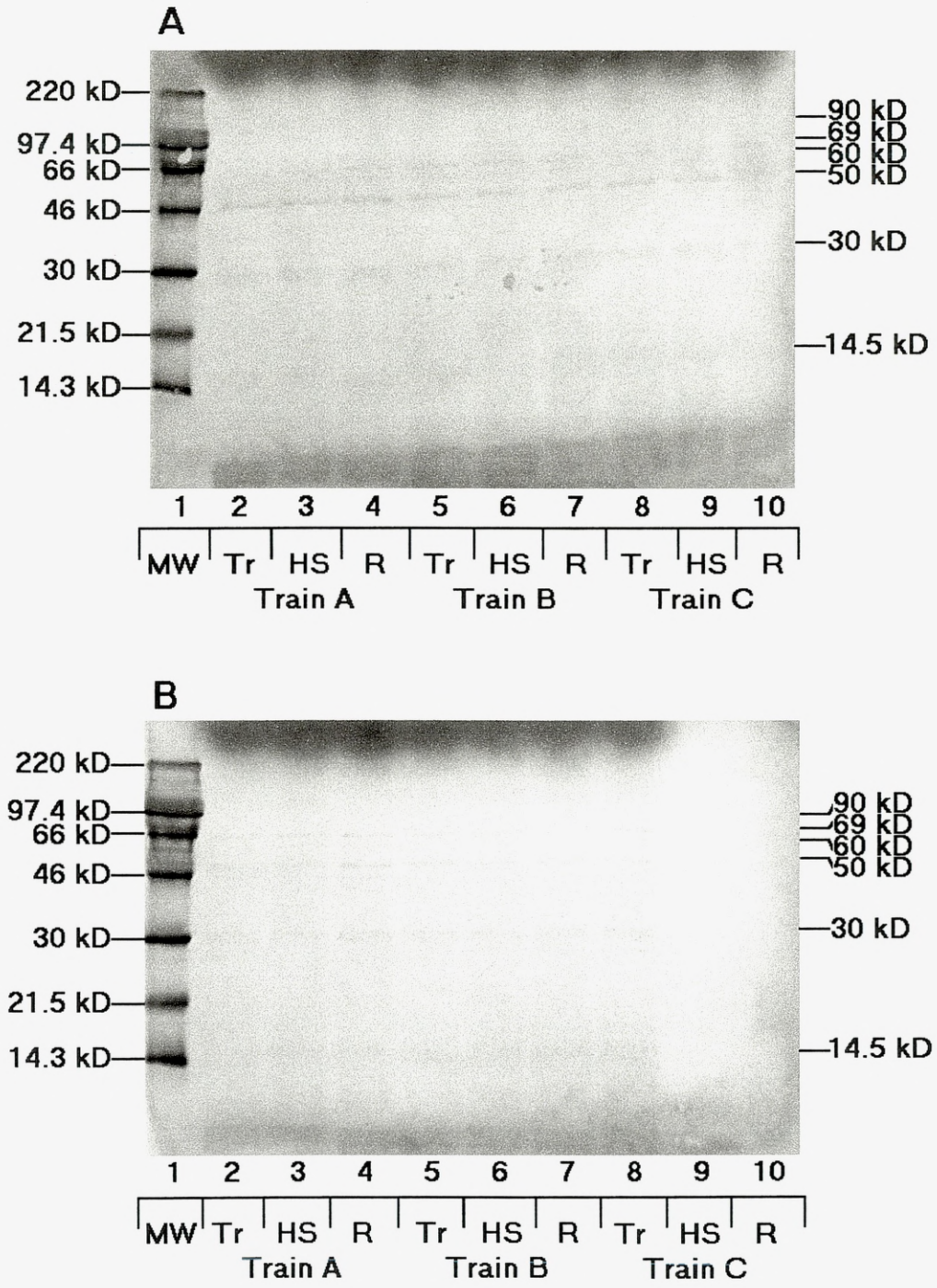


Figure 3.12 A and **B**. Autoradiograms of *in vitro* translation extracts of Bb24 and 12C respectively. Numbers on the right indicate mRNA products expressed during the various temperature regimes imposed. Positions of the molecular weight markers are shown on the right of each gel.

Blot A, Bb24 *in vitro* translation extracts. Lanes 2 - 4 represent Train A cultures: lane 2 - Train A; lane 3 - extreme heat-treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat-treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 - Train C; lane 9 - extreme heat-treatment; lane 10 - recovery.

Blot B, 12C *in vitro* translation extracts. Lanes 2 - 4 represent Train A cultures: lane 2 -Train A; lane 3 - extreme heat-treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat-treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 -Train C; lane 9 - extreme heat-treatment; lane 10 - recovery. Lane 1 on both gels - rainbow high molecular weight markers in the range of 14.3 - 220 kD (Amersham International plc.).

3.2 Immunological Studies to Detect Specific Heat-Shock Proteins and Their Expression.

The main aims of the work were to ascertain the following.

(i) To detect the presence of hsp60 and 70 homologues by polyclonal antiserum and monoclonal antibodies, present in whole cell extracts of Bb24, 12C, *S. cerevisiae* (L-2226) and chick embryo fibroblasts (CEF).

(ii) To make comparisons of antigens present in L-2226 and CEF with those detected in the two *Serpula* species.

(iii) To demonstrate the effect of the various temperature regimes on protein ubiquitination using anti-ubiquitin polyclonal antiserum. This allowed the presence of any proteins ear-marked for degradation, by the well-characterised ubiquitin-mediated proteolytic pathway, to be detected.

(iv) To detect the presence of cross reactive products relating to hsp60 and 70 homologues in rabbit reticulocyte driven *in vitro* translation products derived from *Serpula* species mRNA (Section 3.1.5).

The detection of specific hsps and ubiquitinated proteins by immunological methods was achieved by the use of polyclonal and monoclonal antibodies. The polyclonal antibodies for hsp60 and 70 and control proteins were kindly donated by Dr. A. Mehlert at the University of Dundee. The antisera were raised against *E. coli* GroEL protein (homologue of hsp60), and mycobacterium strain Y3111 hsp70 protein respectively. The monoclonal antibodies to hsp60 and hsp70 and anti-ubiquitin polyclonal antiserum were purchased from StressGen Biotechnologies Incorp. Refer to Appendix A, A1 for specificity of antibodies.

3.2.1 Preliminary Control Experiments to Identify any Non-Specific Binding.

Initial experiments were set out to check for the occurrence of any non-specific binding of the antibodies using control Western blots of Bb24, 12C, yeast and CEF intracellular extracts incubated with NRS (Figure 3.13 A - D). Non-specific binding of a low molecular weight antigen with a molecular weight of approximately 14.3 kD was detected in the control blots for Bb24 and 12C. No corresponding signal appeared in the extracts of L-2226 and CEF.

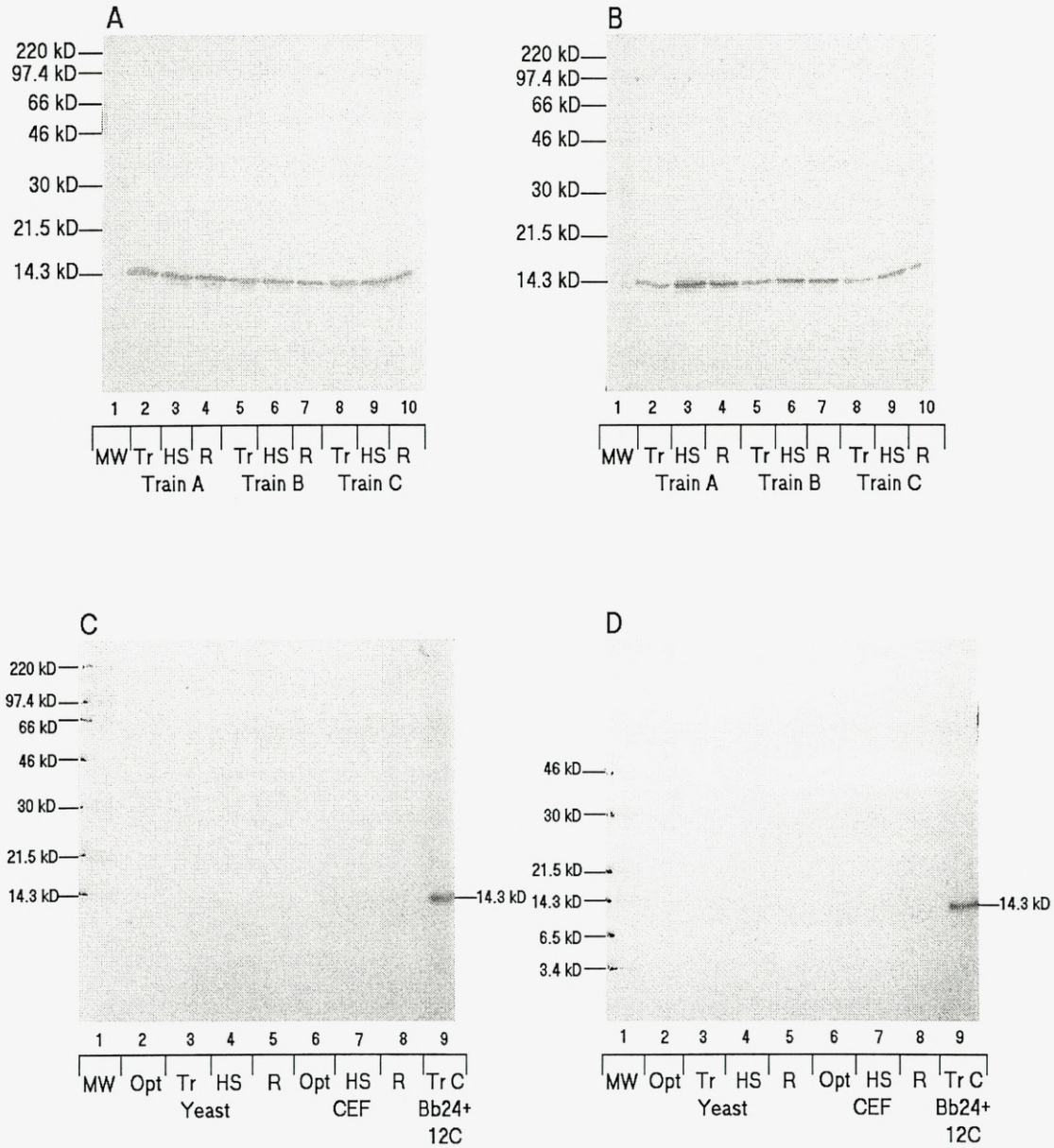


Figure 3.13 A - D. Control Blots of intracellular extracts of Bb24 (A), 12C (B), L-2226 and CEF (C and D) incubated with 1:500 dilution of NRS. Blots A - C incubated with secondary antibodies 1:1000 dilution HRP anti-rabbit IgG, Blot D with 1:1000 dilution HRP anti-mouse IgG. Samples were equalised for protein content of 6 μg and blots were visualised by DAB staining.

Blots A and **B**. Lane 1 - (MW) on both blots - rainbow high molecular weight markers in the range of 14.3 - 220 kD (Amersham International plc.). (A), lanes 2 - 10 represent Bb24 intracellular extracts which have undergone various temperature regimes: lane 2 - 4, Train A cultures: lane 2 (Tr) -Train A; lane 3 (HS) - exposed to extreme heat treatment; lane 4 (R) - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 (Tr) - Train B; lane 6 (HS) - extreme heat treatment; lane 7 (R) - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 (Tr) -Train C; lane 9 (HS) - extreme heat treatment; lane 10 (R) - recovery. (B), 12C intracellular extracts: Lanes 2 - 4 represent Train A cultures: lane 2 Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 -Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 -Train C; lane 9 - extreme heat treatment; lane 10 - recovery.

Blots C and **D**. Lane 1 - (MW) on both gels - rainbow molecular weight markers in the range of (C) 14.3 - 220 kD and (D) 2.35 - 46 kD (Amersham International plc.). Lanes 2 - 5 represent L-2226 extracts which have undergone various temperature regimes: lane 2 - Opt, cultures grown at optimum conditions; lane 3 - Tr, sublethal temperature regime; lane 4 - HS, extreme heat treatment; lane 5 - R, recovery. Lanes 6 - 8 represent CEF extracts which have undergone a heat-shock event: lane 6 - Opt, optimum conditions of 37 ^{0}C / 1 hr; lane 7 - HS, heat-shock treatment of 42 ^{0}C / 1 hr; lane 8 - R, recovery of 37 ^{0}C / 1 hr. Lane 10 - Tr C, represents co-separation of Bb24 and 12C Train C control intracellular extracts.

Figure 3.13 also clearly demonstrates that the non-specific binding at approximately 14.3 kD detected, could represent a protein or proteins with the same surface epitope in both species as the co-separation of Bb24 and 12C Train C extracts in Figure 3.13 C and D results in one antigenic band detected. The phenomenon of this non-specific binding was investigated further to determine whether it was specific to *Serpula* species, or whether it occurred in other basidiomycetes. Cultures of *Coniophora puteana,* grown in the same optimal conditions as Bb24, were exposed to the same Train C treatment regimes. The resultant intracellular extracts were analysed alongside those of Bb24 and 12C to establish if any non-specific binding occurs. Figure 3.14 represents the control blot and it clearly demonstrates that there is no apparent binding with extracts of *C. puteana.*

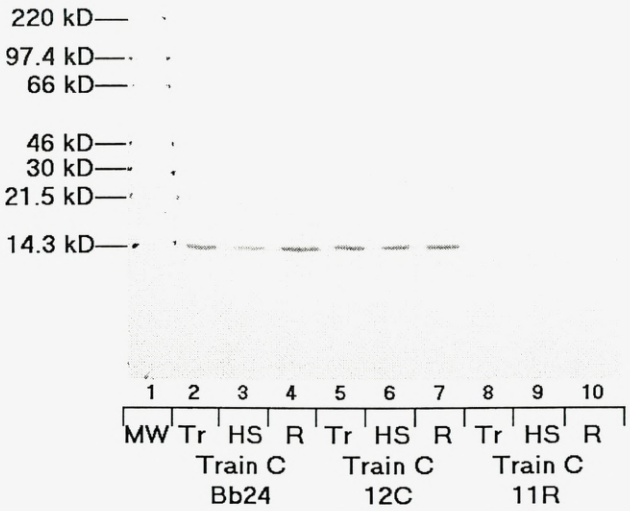


Figure 3.14. Comparison of basidiomycetes, *S. himantioides*, *S. lacrymans* and *C. puteana* for the detection of the presence of cross reactive 14.3 kD band. Control blot was primarily incubated with 1:500 dilution of NRS, followed by 1:1000 dilution with HRP anti-rabbit IgG. Samples were equalised for protein content of 6 μg and blots were visualised by DAB staining. Lane 1 - rainbow high molecular weight markers in the range of 14.3 - 220 kD (Amersham International plc.). Lanes 2 - 4 represent Bb24 intracellular extracts, Train C control cultures: lane 2 -Train C; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent 12C intracellular extracts, Train C control cultures: lane 5 -Train C; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes-8 - 10, represent C. puteana intracellular extracts, Train C control cultures lane 8 -Train C; lane -extreme heat treatment 9; lane 10 - recovery.

3.2.2 Detection of Hsp60 and 70 Homologues in Intracellular Extracts of Bb24 and 12C Using Polyclonal Antiserum.

The appearance of two important molecular chaperones involved in the heat-shock response, namely hsp60 and 70 was investigated *in vivo* in Bb24 and 12C using polyclonal antiserum. Figure 3.15 A and B illustrate the results obtained for the two species, with a distinct difference seen between Bb24 and 12C. A positive signal for an hsp60 homologue is detected by anti-GroEL polyclonal antiserum only in Bb24 intracellular extracts (Figure 3.15 A, lanes 2, 4, 5, 6, 8 and 9). The bands present are smaller in size to that of the control GroEL protein (lane 1). When the resultant autoradiogram for this particular blot was compared to the whole extract profile by superimposing the two profiles, alignment of a 60.6 kD cell extract protein to the blot signal was seen, Figure 3.16 A - D, illustrates the alignment of the profiles.

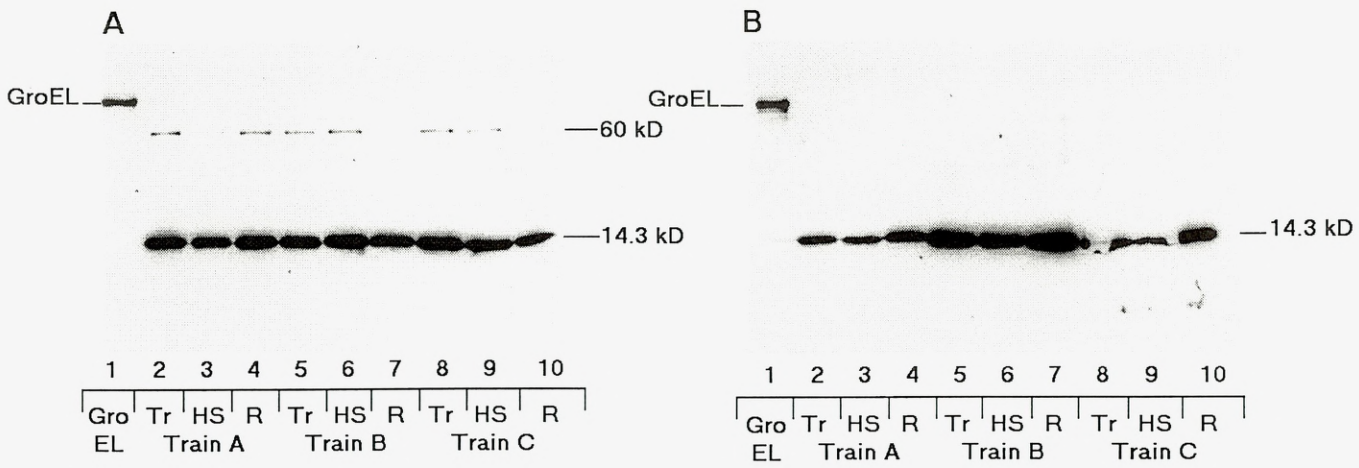


Figure 3.15 A and B. ECL Western blot analysis for the detection of hsp 60 in Bb24 (A) and 12C (B) intracellular extracts of cultures which have undergone various temperature regimes using 1:500 dilution of anti-GroEL serum. Samples were equalised for total protein content of 6 μg.

Blot A, Bb24 intracellular extracts. Lanes 2 - 4 represent Train A cultures: lane 2 - Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 - Train C; lane 9 - extreme heat treatment; lane 10 - recovery.

Blot B, 12C intracellular extracts. Lanes 2 - 4 represent Train A cultures: lane 2 -Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 -Train C; lane 9 - extreme heat treatment; lane 10 - recovery. Lane 1 on both blots - 2.5 μg of GroEL control protein.

Figure 3.16 C is a composite using the ECL blot profile (A) from Figure 3.15 A and its resultant autoradiogram (B). Demonstrating the position of the hsp60 homologue antigenic signal to a band running approximately at 60.6 kD seen in the superimposed autoradiogram (B). The molecular weight of 60.6 kD was deduced from repeating in parallel a second gel with markers (D), therefore establishing the molecular weight of the antigenic signal seen in the ECL blot (A).

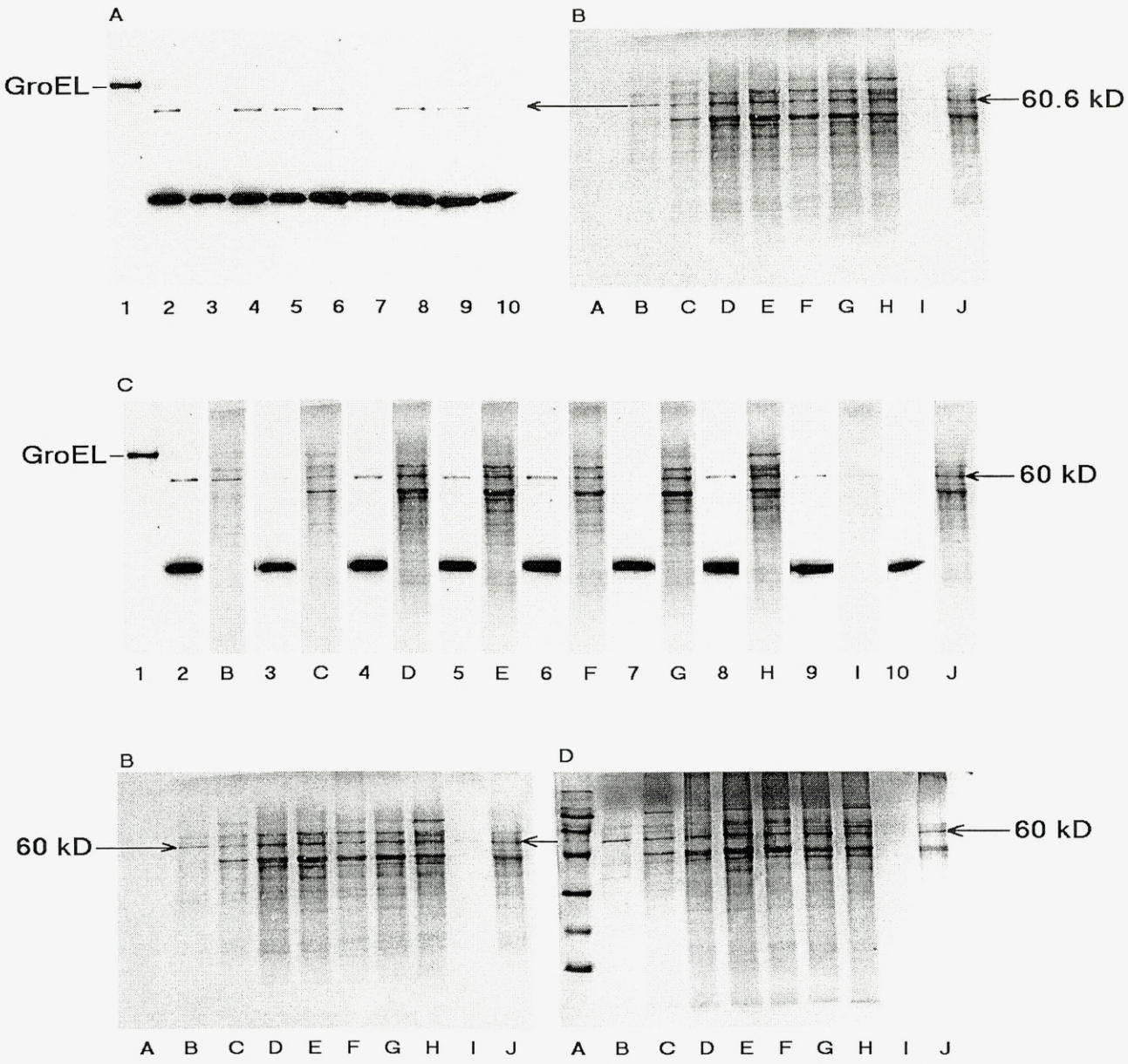


Figure 3.16 A - D. ECL Western blot analysis for the detection of hsp 60 in Bb24 intracellular extracts (A), with the autoradiographic profile of the blot (B). ECL blot aligned against the resultant autoradiographic profile (C) to illustrate the position of hsp60 signal which coincides with a polypeptide of 60.6 kD weight approximately. The 60.6 kD protein was determined from autoradiogram D which represents a repeat run of the samples used in the blot with molecular weight markers present in lane A. Refer to Figure 3.14 B for description of lanes in all the profiles.

Detection of an hsp70 homologue in intracellular extracts of Bb24 and 12C was also investigated by anti-mycobacterium hsp70 polyclonal antiserum (Figure 3.17 A and B). There is no apparent detection of hsp70 homologue in 12C extracts (Blot B), but a relatively strong signal running at the same level as the mycobacterium hsp70 control protein occurs for extreme heat treated and recovery Bb24 control intracellular extracts does occur (Blot A, lanes 9 and 10).

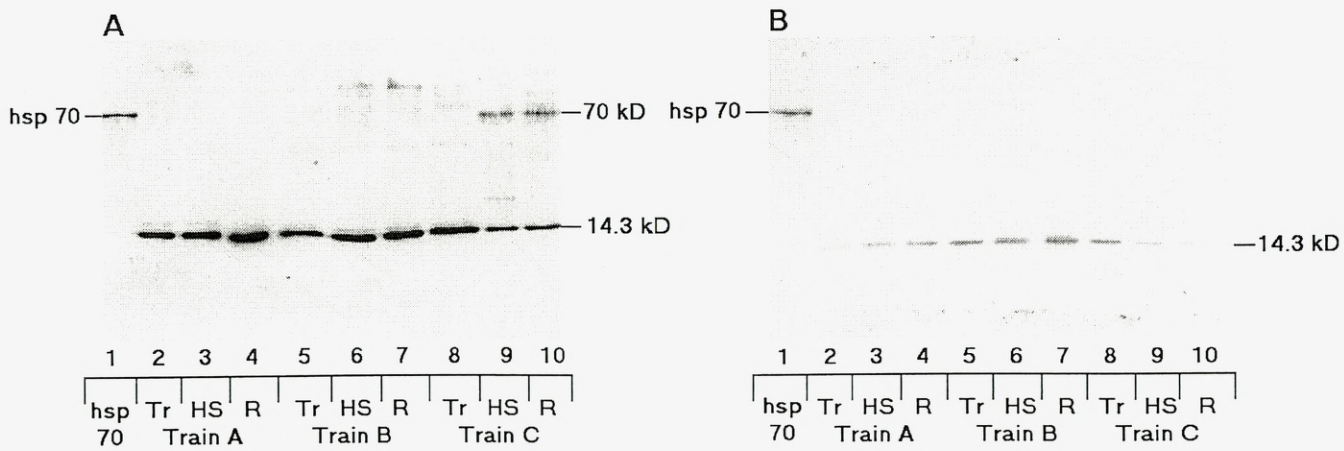


Figure 3.17 A and **B**. ECL Western blot analysis for the detection of hsp 70 in Bb24 and 12C intracellular extracts of cultures which have undergone various temperature regimes using 1:500 dilution of anti-mycobacterium Hsp 70 polyclonal antibody. Samples were equalised for total protein content of 6 µg.

Blot A, Bb24 intracellular extracts. Lanes 2 - 4 represent Train A cultures: lane 2 - Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 - Train C; lane 9 - extreme heat treatment; lane 10 - recovery. **Blot B**, 12C intracellular extracts. Lanes 2 - 4 represent Train A cultures: lane 2 -Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 - Train C; lane 9 - extreme heat treatment; lane 10 - recovery. Lane 1 on both blots - 2.5 µg of mycobacterium hsp70 control protein.

3.2.3 Detection of Hsp60 and 70 Homologues in Cellular Extracts of Bb24, 12C, *S. cerevisiae* and Chick Embryo Fibroblasts Using Monoclonal Antibodies.

Monoclonal antibodies failed to detect hsp60 in Bb24 and 12C cell extracts. Figure 3.18 A and B illustrate that there is no apparent antigen recognition by the monoclonal antibody in either of the two species of *Serpula*.

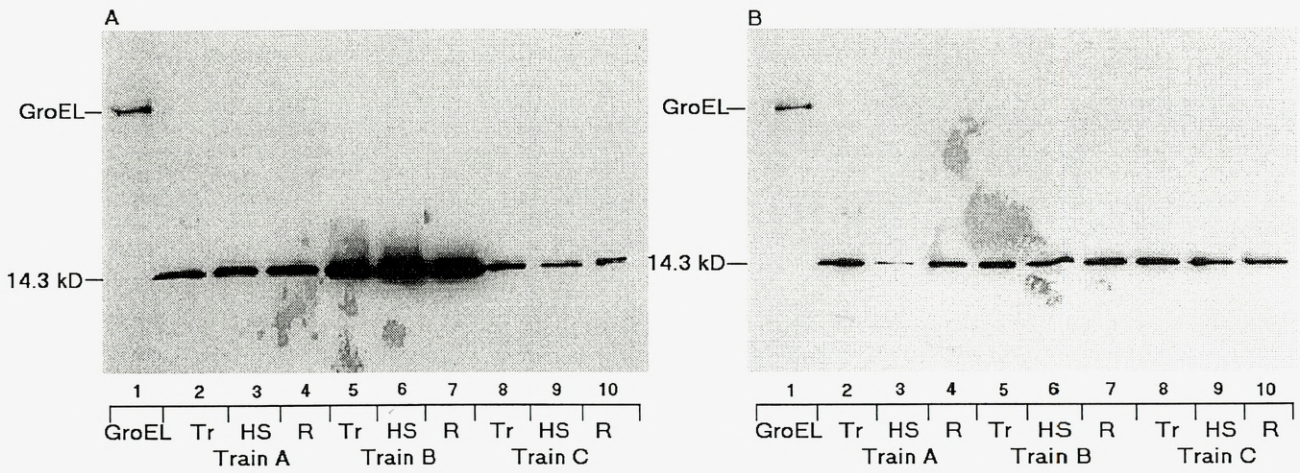
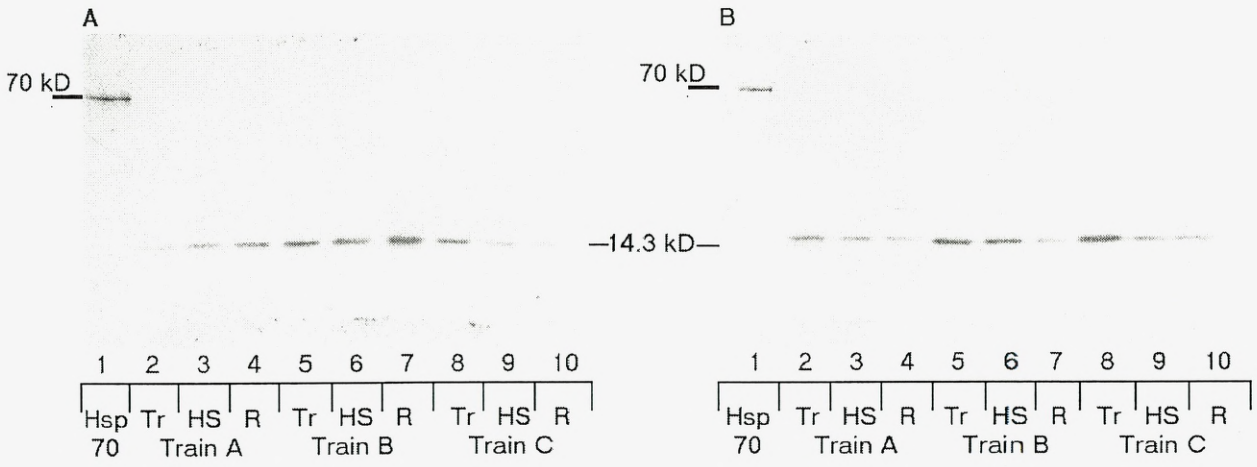


Figure 3.18 A and **B**. ECL Western blot analysis for the detection of hsp60 using 1:500 dilution of anti-60 kD Hsp monoclonal antibody in Bb24 and 12C intracellular extracts of cultures, which have undergone various temperature regimes. Samples were equalised for total protein content of 6 μg.

Blot A, Bb24 intracellular extracts. Lanes 2 - 4 represent Train A cultures: lane 2 - Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 - Train C; lane 9 - extreme heat treatment; lane 10 - recovery. **Blot B**, 12C intracellular extracts. Lanes 2 - 4 represent Train A cultures: lane 2 -Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 - Train C; lane 9 - extreme heat treatment; lane 10 - recovery. Lane 1 on both blots - 2.5 μg of GroEL control protein.

A similar profile was obtained for hsp70 using the anti-Hsp70 monoclonal antibody. The absence of an appropriate antigenic signal for either of the *Serpula* species may be observed in Figure 3.19 A and B.



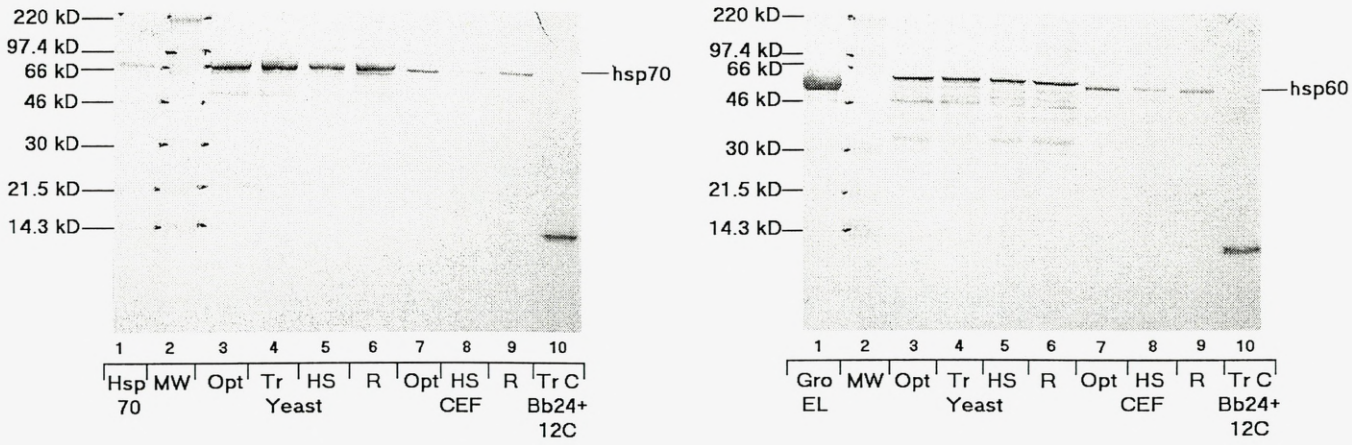
Figures 3.19 A and **B**. ECL Western blot analysis for the detection of hsp 70 in Bb24 and 12C intracellular extracts of cultures which have undergone various temperature regimes using 1:500 dilution of anti-Hsc70/Hsp70 monoclonal antibody. Samples were equalised for total protein content of 6 μg.

Blot A, Bb24 intracellular extracts. Lanes 2 - 4 represent Train A cultures: lane 2 - Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 - Train C (optimum growth conditions); lane 9 - extreme heat treatment; lane 10 - recovery.

Blot B, 12C intracellular extracts. Lanes 2 - 4 represent Train A cultures: lane 2 -Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 - Train C (optimum growth conditions); lane 9 - extreme heat treatment; lane 10 - recovery.

There are a number of possibilities as to why there was no detection of the respective hsps by monoclonal antibodies (MAb's). First, because of the high specificity of the MAb's which only bind to a specific antigenic determinant or epitope, detection might not occur. If such epitopes do not exist on the hsp60 and 70 homologues of the two *Serpula* species, then there will be no reaction of the MAb's with the cellular extracts. Second, the hsp in question may not be expressed in *Serpula* species, although this explanation is unlikely for Bb24 as cross-reactive material was detected by anti-hsp60/GroEL and anti-hsp70 polyclonal antiserum (Figures 3.16 and 3.17, Blot B). By contrast, no cross-reactive products were detected in 12C counterparts by the same antiserum so perhaps 12C do not produce either hsp60 or 70. Finally, post-translational processing of cross-reacting antigens might remove relevant epitopes.

A further experiment was initiated to determine whether the monoclonal antibodies used could detect either hsp60 or 70 in two other, distinctly different eukaryotic systems, namely *S. cerevisiae* (L-2226) and CEF. Figure 3.20 A and B illustrate the detection of homologues of hsp70 and 60 in intracellular extracts of yeast and CEF.



Figures 3.20 A and **B**. Western blot analysis of hsp70 and 60 using monoclonal antibodies (1:500 dilution of anti-Hsc70/Hsp70 and anti-60 kD Hsp monoclonal antibodies, respectively) in yeast (L-2226 wine strain) and CEF intracellular extracts. Samples were equalised for protein content of 6 μg and blots were visualised by DAB staining.

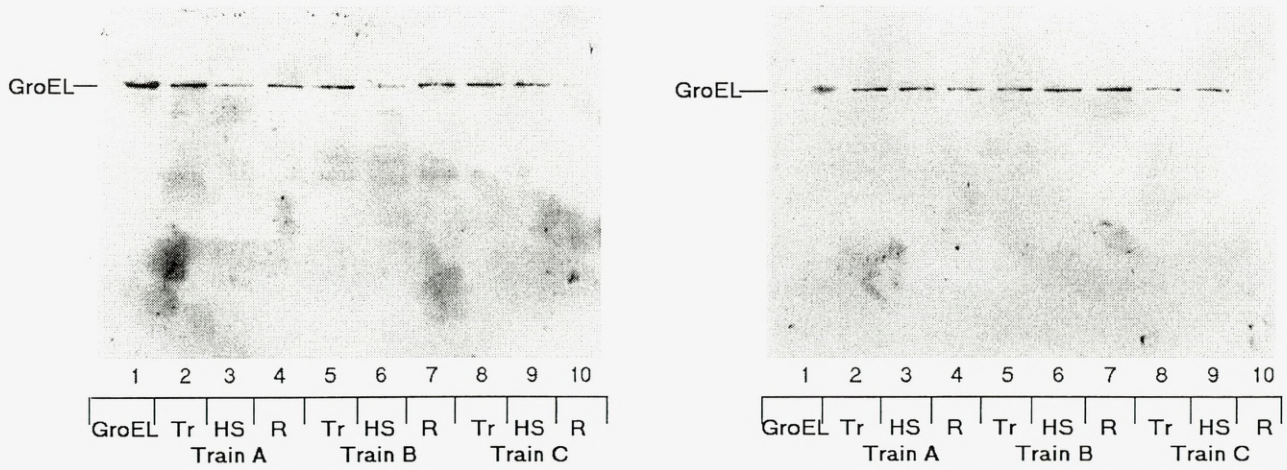
Blots A and **B**. Lane 1 on both blots - 2.5 μg of mycobacterium strain Y3111 hsp70 (A) and hsp60/GroEL (B) control proteins were loaded. Lanes 3 - 6 represent L-2226 extracts: lane 3 - optimum conditions; lane 4 - sublethal temperature; lane 5 - extreme heat treatment; lane 6 - recovery. Lanes 7 - 9 represent CEF extracts: lane 7 - optimum conditions; lane 8 - heat-shock treatment; lane 9 - recovery. Lane 10 - co-separation of Bb24 and 12C Train C intracellular extracts. Lane 2 (MW) on both blots - rainbow high molecular weight markers, 14.3 - 220 kD (Amersham International plc.).

Antigenic signals corresponding in size to the control mycobacterium hsp70 and hsp60/GroEL proteins, were detected the MAb's in both eukaryotic systems. Similar results were also obtained for detection by the polyclonal antiserum (data not shown). Lane 10 in both blots represent a co-separation of both Bb24 and 12C controls (Train C) extracts, again illustrating no apparent signal for either of the hsps present by monoclonal antibody detection.

Although the various hsps are universally conserved throughout the eukaryotic and prokaryotic systems there are slight differences of size in the respective hsp homologues. This can be demonstrated clearly for hsp60/GroEL blot, as the signal for L-2226 runs slightly higher than that of CEF, which runs approximately the same level as the control GroEL protein in lane 1 (Figure 3.20 B).

3.2.4 Detection of Hsp60 and 70 Homologues in Bb24 and 12C *In Vitro* Translation Extracts Using Monoclonal Antibodies.

To determine if the lack of an antigenic signal using MAb's against Bb24 and 12C extracts was due to post-translational processing, the MAb's were tested with the *in vitro* translation products. Figure 3.21 A and B illustrate the results for Bb24 and 12C extracts respectively. The western blots show a signal corresponding to that of the control GroEL protein in lane 1. However, there is no apparent signal in either of the Train C recovery extracts for Bb24 or 12C (lane 10, both A and B blots). The differing sizes of the antigenic signals detected for Bb24 extracts found *in vivo* (Figure 3.18 A) and *in vitro* (Figure 3.21 A) is consistent with post-translational processing. That only the *MAb* detects the in vitro product is consistent with a specific epitope being removed during processing.

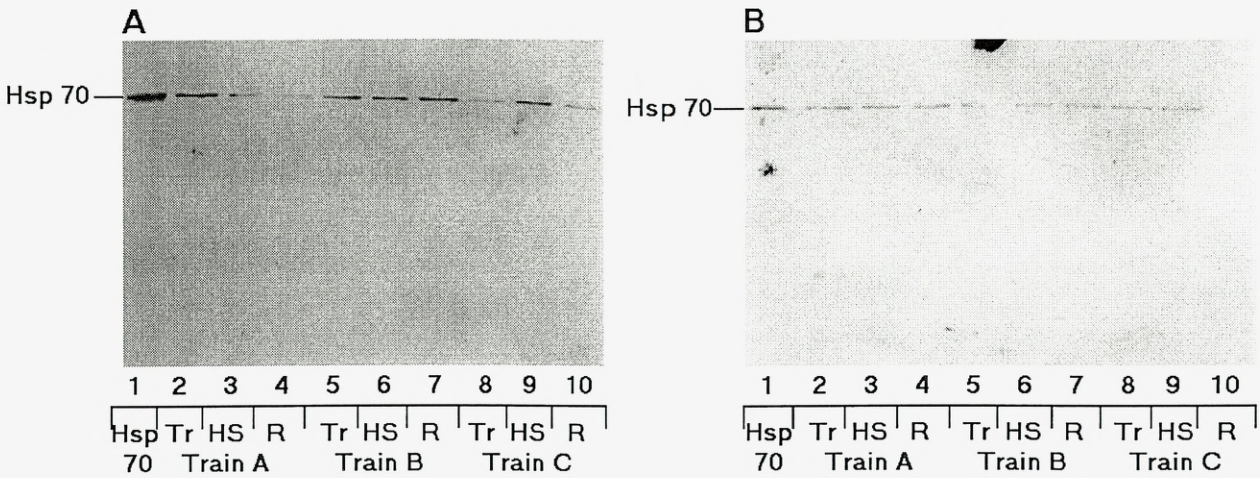


Figures 3.21 A and **B**. ECL Western blot analysis for the detection of hsp 60 in Bb24 and 12C *in vitro* translation extracts cultures which have undergone various temperature regimes using 1:500 dilution of anti-60 kD Hsp monoclonal antibody.

Blot A, Bb24 *in vitro* translation extracts. Lanes 2 - 4 represent Train A cultures: lane 2 -Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 -Train C; lane 9 - extreme heat treatment; lane 10 - recovery.

Blot B, 12C *in vitro* translation extracts. Lanes 2 - 4 represent Train A cultures: lane 2 -Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 -Train C; lane 9 - extreme heat treatment; lane 10 - recovery. Lane 1 on both blots - 2.5 μg of GroEL control protein was loaded.

Similar experiments were undertaken with anti-Hsc70/Hsp70 monoclonal antibody to determine if hsp70 could be detected in *in vitro* translation systems. Figure 3.22 A and B, illustrates the results obtained. A band was detected which runs approximately at the same molecular weight as the control mycobacterium hsp70 protein in lane 1 of both blots. The antigenic signal in Bb24 is stronger and present in all the extracts probed, while there is no identifiable signal present in 12C control recovery extract (lane 10, Figure 3.22 B).



Figures 3.22 A and **B**. ECL Western blot analysis for the detection of hsp70 in Bb24 and 12C *in vitro* translation extracts cultures which have undergone various temperature regimes using 1:500 dilution of anti-Hsc70/Hsp70 monoclonal antibody.

Blot A, Bb24 *in vitro* translation extracts. Lanes 2 - 4 represent Train A cultures: lane 2 -Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 -Train C; lane 9 - extreme heat treatment; lane 10 - recovery. **Blot B**, 12C *in vitro* translation extracts. Lanes 2 - 4 represent Train A cultures: lane 2 -Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 -Train C; lane 9 - extreme heat treatment; lane 10 - recovery. Lane 1 on both gels - 2.5 μg of mycobacterium strain Y3111 Hsp 70-control protein was loaded.

3.2.5 Detection of Proteins Labelled for Degradation: Protein Ubiquitination.

A further aspect of the heat-shock response is the ubiquitin system, a multicomponent, ATP-dependent pathway, whereby ubiquitin conjugates to abnormal proteins or proteins with a high turnover rate, ultimately leading to their degradation. Due to the highly conserved nature of ubiquitin, an anti-ubiquitin polyclonal antibody could be used to investigate any changes in the patterns of protein ubiquitination in heat-treated and control Bb24 and 12C cultures (Figure 3.23 A and B).

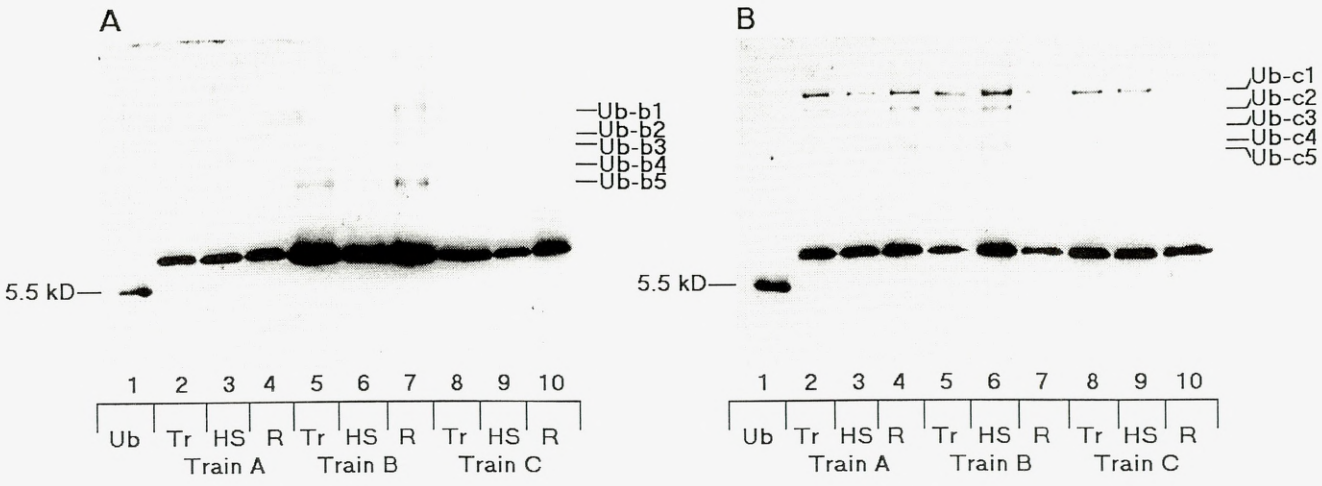


Figure 3.23 A and **B**. ECL Western blot analysis for the detection of ubiquitin conjugated proteins in Bb24 and 12C intracellular extracts of cultures which have undergone various temperature regimes using 1:500 dilution of anti-Ubiquitin polyclonal serum. Samples were equalised for total protein content of 6 µg. Figures Ub-b1 to b5 and Ub-c1 to c5 to the left of the blots highlight ubiquitinated conjugates of Bb24 and 12C respectively.

Blot A, Bb24 extracts. Lanes 2 - 4 represent Train A cultures: lane 2 -Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 -Train C; lane 9 - extreme heat treatment; lane 10 - recovery.

Blot B, 12C extracts. Lanes 2 - 4 represent Train A cultures: lane 2 -Train A; lane 3 - extreme heat treatment; lane 4 - recovery. Lanes 5 - 7 represent Train B cultures: lane 5 - Train B; lane 6 - extreme heat treatment; lane 7 - recovery. Lanes 8 - 10 represent Train C control cultures: lane 8 -Train C; lane 9 - extreme heat treatment; lane 10 - recovery. Lane 1 on both gels (Ub) - 5 μg of bovine ubiquitin control protein (Sigma, cat. U6253).

Proteins highlighted by their conjugation to ubiquitin are detected for both Bb24 and 12C extracts (Figure 3.23 A and B, respectively). The distribution and intensity of the bands illustrate differences between both species. For Bb24 ubiquitinated proteins detected, are in mid to low molecular weight range compared to 12C where they are distributed in the high to mid range. The proteins detected in Bb24 are also restricted primarily to Train B extracts (lane 5 - 7), compared to 12C, which are present in most of the extracts with the exception of Train C, recovery extract (lane 10). The intensity of the bands highlighted overall are feint for both species, with the exception of two bands (depicted as Ub-c1and Ub-c2) displayed in the 12C blot (Figure 3.23 B). These bands represent relatively high molecular weights, ranging between 60 - 90 kD. The band Ub-c1 shows interesting quantitative variation in that it is greatly reduced in the recovery phase of both 12C Train B and C samples (lanes 7 and 10 respectively).

3.2.6 Summary and Concluding Remarks.

The non-specific binding of the band running approximately at 14.3 kD in intracellular extracts of Bb24 and 12C occurred regardless of the type of secondary detection system used. Vigrow (1992) also detected this same phenomenon in immuno-dot blot control assays, and in resultant Western blots for a range of *S. lacrymans* and *S. himantioides* isolates. It was interesting to note that this non-specific reactivity did not occur in the *in vitro* translation extracts as well as in *S. cerevisiae*, L-2226 and CEF systems (Figure 3.13 C and D). It was also clearly demonstrated through co-separation of Bb24 and 12C extracts (Figure 3.20 A and B) that the non-specific binding detected could represent a protein or proteins with the same surface epitope in both species.

The question of whether this was a unique feature to *Serpula* species was investigated by analysing intracellular extracts of another basidiomycete *Coniophora puteana*. It was illustrated that this cross reactivity did not occur in *C. puteana*, suggesting that this might be the case. This can only be fully verified by systematic tests over a wide variety of basidiomycetes, investigating the presence of this lower molecular weight band.

The immunological detection of important hsps in both *Serpula* species, as well as two other diverse eukaryotic systems, yeast and CEF are summarised in the following table.

Hsp Antibodies	Intracellular				*In Vitro*	
	12C	Bb24	Yeast	CEF	12C	Bb24
Anti-GroEL	-	(+)	+	+		
Anti-myco 70	-	+	+	+		
Anti-ubiquitin	+	+				
MAb 60	-	-	+	+	+	+
MAb 70	-	-	+	+	+	+

Table 3.2.6. Immunological Detection of Possible hsps in intracellular extracts of Bb24, 12C, yeast (L-2226), CEF and *in vitro* translation extracts of Bb24 and 12C. '+' and '-' represents positive and negative signals respectively. (+) for intracellular Bb24 extract represents signal detected which is lower in molecular weight to that of the control hsp60/GroEL protein. '+' in bold refers to data not shown.

From the above summary illustrated in Table 3.2.6, a number of points can be made.

(i) Both MAb's and the polyclonal antiserum detected signals for hsp60 and 70 in the L-2226 and CEF extracts. Indicating that the test systems were operating efficiently.

(ii) An hsp60 homologue was detected using the respective polyclonal antiserum in Bb24, which had a lower molecular weight than that of the control. No corresponding antigen was detected for 12C. Again, hsp70 was only detected in Bb24 extracts. These observations are consistent with those from Section 3.1.3, it was concluded that a protein related in molecular weight to hsp70 was present in intracellular extracts of Bb24, but there was no identifiable counterpart in 12C extracts.

(iii) Although no signals were identifiable for both the hsp60 and 70 using monoclonal antibodies on intracellular extracts of Bb24 and 12C, a signal was identified in their *in vitro* translation counterparts. The loss of detection of hsp60 and 70 by MAb's in the cell extracts of both *Serpula* species implies that both (a) undergo some measure of post-

translational modification in which the epitope recognised by the MAb's is removed or (b) the proteins are rapidly degraded in the *in vivo* systems.

(iv) Ubiquitination of proteins was detected in both intracellular extracts of Bb24 and 12C (Figure 3.23 A and B). The molecular weight ranges of the predominant ubiquitin-conjugated proteins were different in the two species. Two signals of high molecular weight were detected in 12C, which was not apparently present in the Bb24 extracts. The position of these bands visually corresponds between the 60 - 90 kD range.

3.3 Molecular Studies to Identify a Putative Hsp70 Gene.

Reverse transcription (RT) followed by polymerase chain reaction (PCR) amplification is a widely used, highly sensitive method for analysis of the expression of low abundance messenger RNA's (Kawaski et. al., 1998). The overall aim of the work was to detect hsp70 mRNA from the two *Serpula* species by RT-PCR with specific identification of the PCR generated products by cloning and sequencing. The following points outline the steps involved in achieving the above aims.

(i) To design a set of short sequence-specific primers from a closely related basidiomycete to the *Serpula* species, namely *A. bisporus* hspA (hsp70) gene sequence (Accession no. X98508).

(ii) To use the sequence specific primers to generate PCR products from genomic DNA isolated from *A. bisporus* (used as a positive control), Bb24 and 12C.

(iii) To use the same primers to generate cDNA products generated by RT-PCR from cellular RNA isolated from *A. bisporus* (used as a positive control), Bb24 and 12C.

(iv) To clone and sequence the amplimers produced by PCR and RT-PCR.

3.3.1 Primer Design.

The design of the primers was achieved by the use of the Wisconsin Genetics Computer Group Sequence Retrieval System (SRS), as described in Chapter 2, Section 2.9.2. The highest percent similarity of known base-pair matches between a number of different fungi was initiated. Table 3.2.7 illustrates the gap comparison of percentage similarity of *A. bisporus* to *Puccinia graminis* and *S. cerevisiae.*

Fungus and sequence length	Percentage similarity of bp regions of *A. bisporus* compared to different classes of fungi	
	1-246 bp	303-536 bp
P. graminis **1-3548 bp**	68.293	65.385
S. pombe **1-2367 bp**	51.220	52.814
S. cerevisiae **1-1981 bp**	44.715	44.017

Table 3.2.7. Gap comparison of the percentage similarity of base pairs, of *A. bisporus* to *Puccinia graminis* and *S. cerevisiae.*

It is clear that related fungi such as *A. bisporus* and *P. graminis* which are classified in the same sub-division or group, Basidiomycotina, but from different classes, Basidiomycetes and Teliomycetes respectively, have the highest bp similarity. Cross referencing the sequences of *A. bisporus* to *S. cerevisiae* and *S. pombe* both from the Ascomycotina family, resulted in a decrease in percentage bp similarity of the sequences investigated *A. bisporus* is the most closely related to the *Serpula* species as they are both basidiomycetes. Synthetic oligonucleotides were designed to amplify the DNA and mRNA sequences related to the heat-shock protein 70 kD isolated from the partially sequenced hspA gene of *A. bisporus* (Schaap et. al. 1996). The *A. bisporus* sequence contained an intron of approximately 50 bp, the designed primers spanned the region before and after this intron allowing easy identification of the genomic DNA and mRNA products (Refer to Chapter 2, Section 2.8.2).

3.3.2 Optimisation of RT-PCR Reaction.

Preliminary experiments were set out to determine the optimum conditions required for efficient one tube, two enzyme PCR reaction. Figure 3.24 summarises the outcome of these preliminary experiments.

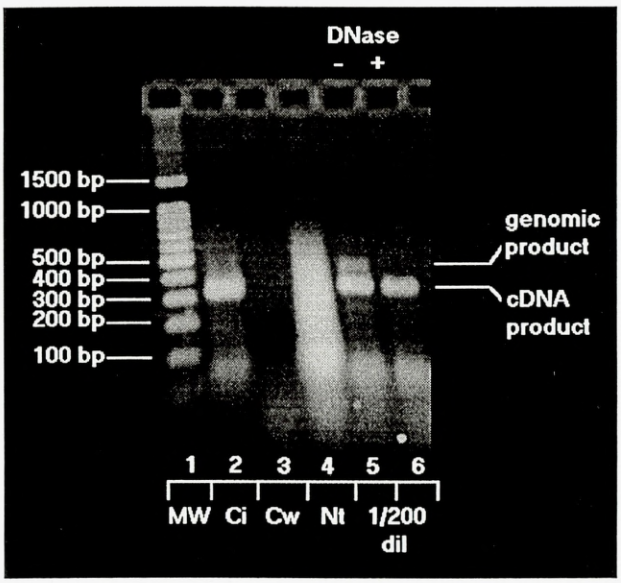


Figure 3.24. RT-PCR of *A. bisporus* mushroom RNA. A neat (lane 4) and a 1/200 dilution (lanes 5 and 6) of mushroom cDNA (before and after DNase treatment, respectively) were amplified by PCR. The genomic and cDNA products denote the positions of the 407 bp and 352 bp fragments respectively. Positive control RNA (lane 2, Ci-internal control) gave a specific amplimer of 323 bp with control oligonucleotide primers provided with the Promega Access RT-PCR system. A negative control (lane 3, Cw-water control) with nuclease-free water substituted for mushroom RNA to ensure there was no contamination of reaction mixture. Lane 1, MW - molecular weight markers, Promega's 100 bp DNA Ladder. Refer to Chapter 2, Section 2.8.2 for approach used to ascertain molecular weights of genomic and cDNA amplimers.

Four important steps were included: - (i), an internal positive control reaction (supplied with Promega's Access RT-PCR kit) was run to ensure that the supplied system was running optimally, producing an amplimer of 323 bp was incorporated (lane 2). (ii), a negative control, whereby water is substituted for the RNA sample in the reaction, to check for any contamination in the reaction mixture. (iii), optimisation of sample concentration by the addition of neat and 1:200 dilution, illustrating that high concentrations of RNA can effectively inhibit the reaction resulting in smearing (lane 4). (iv), the elimination of trace amounts of DNA present in the total RNA extracts was achieved by digestion of the total RNA samples with Promega's RQ1 RNase-Free DNase (lane 5 - no DNase, lane 6 - with DNase). (v), the integrity of the RNA was protected from possible degradation by the addition of RNasin Ribonuclease Inhibitor (Promega) to the reaction mixture.

3.3.3 Generation of Genomic and cDNA Products by RT-PCR and PCR Analysis.

Using the gene specific primers, 407 bp DNA and 352 bp cDNA fragments were amplified by PCR and RT-PCR respectively from both species of *Serpula* and *A. Bisporus* as the control. Figures 3.25 and 3.26 illustrate the RT-PCR products for 12C and Bb24 respectively. A clear signal can easily be detected in all the 12C Train A extracts (lanes 4 - 6), and the extreme heat treated extracts of Train B and C (lane 8 and 11) which run at the same level to that of the mushroom control in lane 2, at 352 bp (Figure 3.25). Feint signals are also detected in the remaining 12C extracts (lanes, 7, 9 10 and 12).

Figure 3.25. Comparison of *A. bisporus* and *S. lacrymans* (12C) products amplified by RT-PCR. Total RNA prepared from the *Agaricus* mushroom and 12C were employed for cDNA synthesis using 50 pmol each of Nat 1 and Nat 2 primers. Lane 1 - MW, 100 bp molecular weight ladder (Promega). Lane 2 - M, *A. bisporus* mushroom, positive control, Lane 3 - Ctrl, negatives control. Lanes 4 - 12 represent 12C samples, which have been, subjected to the different training/sublethal (Tr), extreme heat (HS) and recovery (R) regimes. Lane 4 - 6 represent Train A cultures (28 ^{0}C/ 24 hrs): 28 ^{0}C/ 24 hrs (lane 4); exposed to extreme heat of 40 ^{0}C/ 2 hrs (lane 5); placed into recovery at 22 ^{0}C/ 24 hrs (lane 6). Lanes 7 - 9 represent Train B cultures (25 ^{0}C/ 48 hrs): 25 ^{0}C/ 48 hrs (lane 7); exposed to extreme heat of 40 ^{0}C/ 2 hrs (lane 8); placed into recovery 22 ^{0}C/ 24 hrs (lane 9). Lanes 10 - 12 represent Train C control cultures (22 ^{0}C/ 24 hrs): 22 ^{0}C/ 24 hrs (lane 10); exposed to extreme heat of 40 ^{0}C/ 2 hrs (lane 11); placed into recovery 22 ^{0}C/ 24 hrs (lane 12). Refer to Chapter 2, Section 2.1.1.2, Figure 2A for schematic of temperature regimes used in the study.

A different picture for the analysis of Bb24 RNA extracts is seen in Figure 3.26. Strong signals also of similar bp size to the mushroom control (lane 2) are detected in the extreme heat treated Train A and recovery Train B extracts (lanes 4 and 8 respectively). Less prominent signals are produced in the remaining lanes with the exception of Train B, extreme heat-treated extracts (lane 7), Train C control and recovery extracts (lanes 9 and 11) with no apparent signal detected.

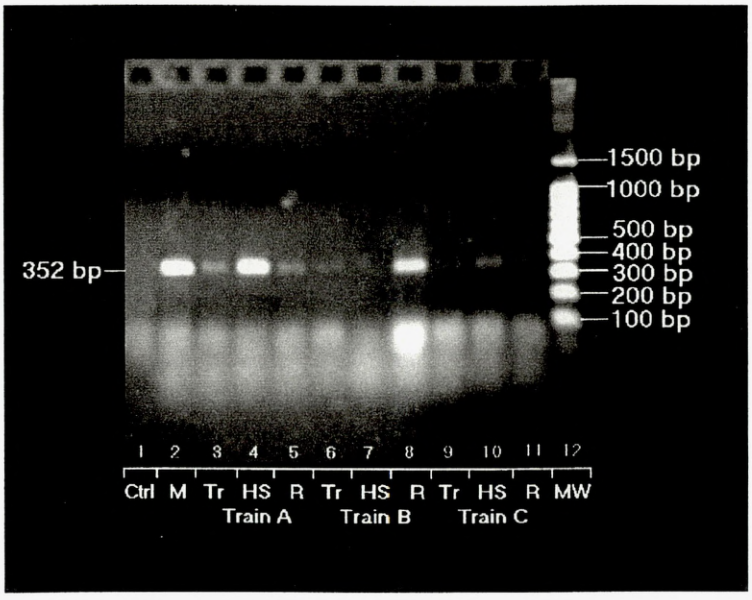


Figure 3.26. Comparison of *A. bisporus* and *S. himantioides* (Bb24) products amplified by RT-PCR. Total RNA from the *Agaricus* mushroom and 12C were employed for cDNA synthesis using 50 pmol each of Nat 1 and Nat 2 primers. Lane 1 - Ctrl, control run. Lane 2 - M, *A. bisporus* mushroom. Lanes 3 - 11 represent Bb24, which have been subjected to the different training/sublethal (Tr), extreme heat (HS) and recovery (R) regimes. Lane 3 - 5 represent Train A cultures (28 ^{0}C/ 48 hrs): 28 ^{0}C/ 48 hrs (lane 3); exposed to extreme heat of 40 ^{0}C/ 3 hrs (lane 4); placed into recovery 25 ^{0}C/ 24 hrs (lane 5). Lanes 6 - 8 represent Train B cultures (30 ^{0}C/ 48 hrs): 30 ^{0}C/ 48 hrs (lane 6); exposed to extreme heat of 40 ^{0}C/ 3 hrs (lane 7); placed into recovery 25 ^{0}C/ 24 hrs (lane 8). Lane 9 - 11 represent Train C control cultures (25 ^{0}C/ 24 hrs): 25 ^{0}C/ 24 hrs (lane 9); exposed to extreme heat of 40 ^{0}C/ 3 hrs (lane 10); placed into recovery 25 ^{0}C/ 24 hrs (lane 11). Refer to Chapter 2, Section 2.1.1.2, Figure 2A for schematic of temperature regimes. Lane 12 - MW, 100 bp molecular weight ladder (Promega).

It is clear that the cDNA products produced are generated from the RNA extracts prepared and is not an aberration from a contaminant, as the water controls in both experiments were clean, i.e. with the absence of bands (lanes 3 and 2, Figures 3.25 and 3.26 respectively). But it is not clear as to the relationship between the variations in intensity of the bands produced in both Figures 3.25 and 3.26. The variations in band intensities from the different samples, may be caused by quantitative variations in the starting material, although there were difficulties in quantifying this material as it was below the level of sensitivity of the Vis/UV spectrophotometer used.

To ensure that the RT-PCR products produced were not from spurious genomic DNA, Figure 3.27 illustrates the ease in identification between the two types of products generated due to the careful design of the primers. The genomic DNA are clearly seen to be greater in size to that of the cDNA counterparts with a predicted and observed 50 bp difference.

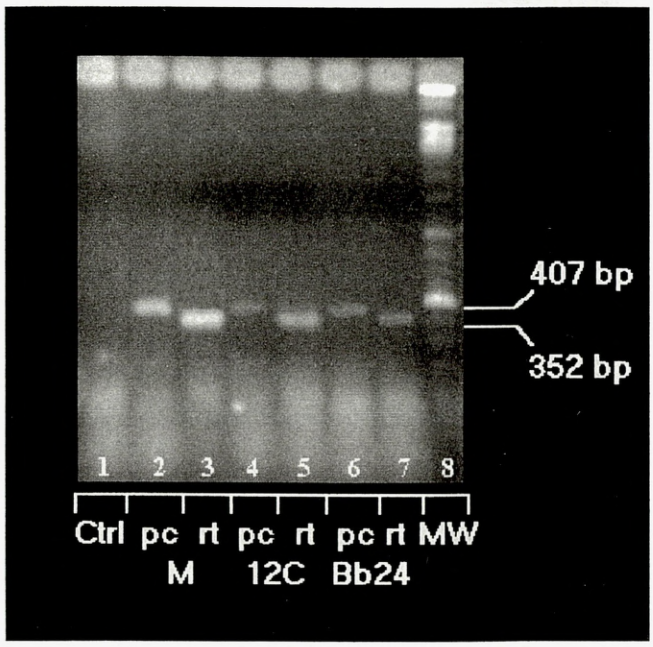


Figure 3.27. Comparison of genomic DNA and cDNA products of *A. bisporus* mushroom and *Serpula* species, 12C and Bb24. Lane 1 - Ctrl, negative water control. Lanes 2 and 3 represent *A. bisporus* DNA and RNA derived products; lane 2 - M pc, mushroom genomic DNA and lane 3 - M rt, mushroom cDNA. Lanes 4 and 5 represent products derived from Train C extreme heat treated extracts of 12C DNA and RNA respectively: lane 4 - 12C pc, 12C genomic DNA produced from Train C; lane 5 - 12C rt, 12C cDNA product. Lanes 6 and 7 are products derived from Train C extreme heat treated extracts of Bb24 DNA and RNA respectively; lane 6 - Bb24 pc, Bb24 genomic DNA from Train C extreme heat treated cultures; lane 7 - Bb24 rt, cDNA product. Lane 8 - MW, 500 bp molecular weight ladder (Appligene).

3.3.4 Cloning and Sequencing of cDNA and Genomic DNA Products.

The purified cDNA and genomic products generated from the previous experiments were ligated into pGEM-T vectors and subsequently used to transform MAX Efficiency DH5α™ competent cells. The following Figure 3.28 represents a plate of cultures with both recombinant and non recombinant colonies (white and blue colonies respectively).

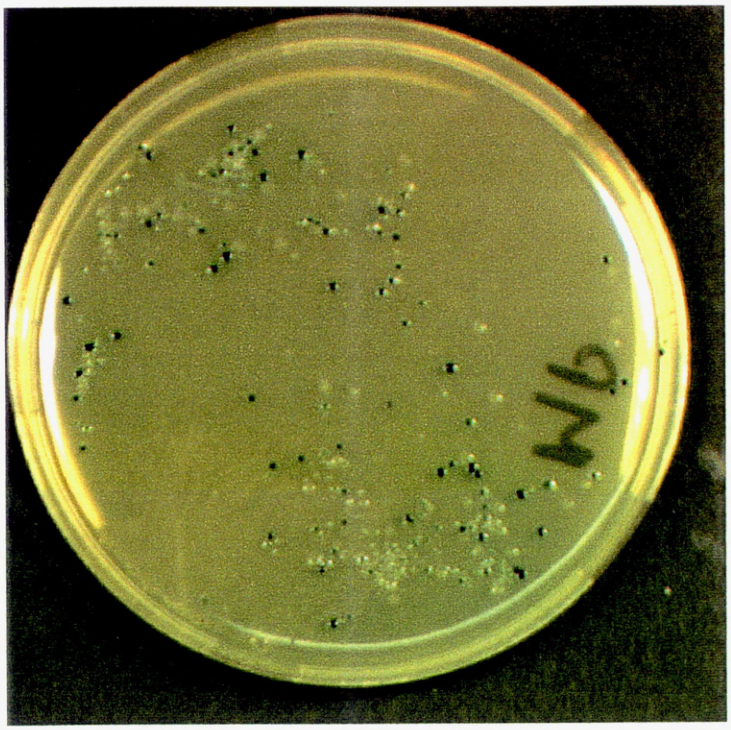


Figure 3.28. White and blue colonies of recombinant and non-transformed clones respectively.

The bacterial plate-containing X-gal represents the effective screening of blue and white colonies. Cloning with Promega's pGEM-T vector involves the insertional inactivation of the *lacZ'* gene; recombinants being distinguished by their inability to synthesize *ß*-galactosidase, resulting in white colonies.

Using the restriction sites of EcoRI within the pGEM-T vector, the inserted cDNA and genomic DNA fragments generated from the RT-PCR and PCR reactions, respectively were sequenced giving the following results (N, signifies unknown base pair). The plasmid yield containing the cDNA fragments derived from RT-PCR reactions from *A. bisporus* and Bb24 was insufficient to give sequencing results (Figures 3.29 - 3.32).

```
  1  TTCCCCCAAA ATTGCCGGCT TTAGGGCCCG ATGTCACATG CNCCCGGCCG
 51  NCANGGCGGC CGNGGGAATT CGATTCACTC ATCTTGGTGG TGAGGATTTT
101  GACAATGCGC TTCTCNANCA CTTCAAGAAC NAGTTCAAGA ANAAGACCAA
151  GTTTGATATC TCTGATGATG CTCGCGCTCT CAGGCGTCTC AGGTCTGCCT
201  GCGAGCGTGC CAAGAGGACT CTCTCCAGTG TCACACAGAC TATGGTCGAG
251  GTTGACTCCC TGTATCAGGT CCGTTAACGA TTTGCTTNTA CNGGTGTCNN
301  CAGCCAGCTG ATGATCTAAT TAGGGCGAAN ACTTCTCTGC TANCATCACC
351  CGTGCTCNTT TCGAGGAGAT TNACGCTGTC CTCTTCAAGT CTACCCTTGA
401  ACCCGNTGAG AAGGTCTTGA AGGATGCNCN NATGGCACGT GAGAAGGTTG
451  ATGACATCCT CCTTGTCGGT GGTTCCNCCC NTATNNTCAC TATTNAATTC
501  CCGGCCGCCT GCNNGTCNAC CATNTGGGAN AGCTCCCCNC GCNTNGGATG
551  CCTAGCTTGA GTTTNCTATT TTGTCCCTAC TAGCTTGGCG TCNCCNGGTC
601  ACAGCNGNTT CCTNTGTNAA ATTGTTATCC CNCCCCATTC CANCCCATNC
651  NAACCNNAAN CCTCNATTTT TTCCNCGNNG TTGCNTAATT NTTCNNCTTN
701  CTCCCTCCTT CGCNTTGCNC TCCNTCCCCC TTTCTTTCTG GNAAACCTTT
751  CNCCNCCTNC CCNNATNAAT CCCCCNTCCC GGGAANNGCT GTTTCCTNTT
801  TCNCCCCCTN TCCTCCTTCC CACTCNCCCC TCTCCCNGTT TTTNNCNNTN
851  CANCNNCTCT CTCNCCCACG CNGTNCTCTC C
```

Figure 3.29. *A. bisporus* (Mp2) genomic DNA sequence, length 881 nucleotides long.

```
  1  TTTTCCCCCA CCTTTGCCCA CTTATAGGCC CGGCTTTTAA NGGCGCGACG
 51  TCGCANGCCC CCGGCCGAAA TGGCGGCCNT GGGAATTCGA TTATACGGGT
101  GGAACCACCG ACGCGCCCTC ACCACCAANA TGAGTGAATC ACTATTGAAT
151  TCGCGGCCGC CTGCAGGTCG ACCATATGGG ANAGCTCCCA ACGCGTTGGA
201  TGCATAGCTT GAGTATTCTA TAGTGTCNCC TAAATAGCTT GGCGTAATCA
251  TGGTCATAGC TGTTTCCNGT GTGAAATTGT TATCCGCTCA CAATTCCACA
301  CNACATANNA GCCGGAANCA TAAANTGTNA AGCCTGGGGT GCCTANTGAN
351  TNATCTAACT CACATNANTT GCGTTGCGCT CACTGNCCGC TTTCCANTCG
401  GGAAACNGTC CTNCCANCTG NATTANTGAT TCTGCCNACT CCCGGGGAGA
451  GGCGGNTTGC GTNTTGGGNG CTCTTCCNCT TCCCNCTCAC TNATTCNCTG
501  CNCTCGGNCN TTCGGTTNNC GCGTANCGGT NTCCNCCCCC TTTANNGCGG
551  TATNACTGTT NTCCACNCAA TCCTGGGATT TCCCCCGAAA TTAACNTGTN
601  NTCTTTACGC CTCNTCCCTG CCNNNTANNC TGTTCAANGG NNNCGTNNCT
651  GNTTTTTTTC NTNCGGTNCC CTCCCNCGAC TANCTATTCC TTAATCCANG
701  CTCACTCCTN AGTGGCGACC CTCTTCTNGC ACTTNCATTA TCCCNCNCTC
751  NCCNCCTGGN ANCNCCTCCT ATTTCTTCTT TCCTCCCCTC CTTNAACTTA
801  CCTTTTCCCC TTTNTCCCTN CTGNAANCTT CTCTCTCTCN TCCCNNCCCT
851  CCCCNTTTTT CTCTTTANTC NTTGNTCTTC CNTCCATCTC NGNCNNNTCN
901  CCC
```

Figure 3.30. Bb24 (Bp4) genomic DNA sequence, length 903 nucleotides long.

```
  1  NCCCCCCCTT TTNCCACCAT TTGCCCGGCT TNANGGCCCG ATTTNNCATG
 51  CNCCCGGCCG NAANGGCGGC CNTGGGAATT CGATTATACG GGTGGAACCA
101  CCGACCACCC TGGGAACTGG TCCTCACCAC CAANATGAGT GAATCACTAA
151  TGAATTCGCG GCCGCCTGCA GGTCGACCAT ATGGGANAGC TCCCAACGCG
201  TTGGATGCAT AGCTTGAGTA TTCTATANTG TCACCTANAT AGCTTGGCGT
251  AATCATGGTC ATAGCTGTNT CCTGTGTGAA ATTGTTATCC GCNCACAATT
301  CCACACNANA TACNAGCCGG AAGCATAAAN TGTNAAGCCT GNGGTGCCNA
351  TTGAGTGAGC CANCTCACAT TAATTGCGTT GCNCTCACTG CCCGCTTTCC
401  ANTCNGGAAA CCNGTCNTGC CAGCTGCNTT AATGAATCTG CCAACGCGCG
451  GGGTACAGGC GGTTTGCGTA NNGGGCNCTC TTCCNCTTCC TCGCTCANTG
501  ACTCTCNGCG CTCGGTCGTT CGGCTGCGGN NATCNGTATC NTCTCTCTCA
551  NAGGNNNGCN ATACCGTTAT CCCANATTCT CGGGATNNCC CCTGAANGAA
601  CNTTTCACCA TAACGTCCNC CNACGCCNCN AACCCTTCNC TGCCTCTTNC
651  CGNCTTTTCT CCNTATGCNC NCCCCCCCTN ACCACNTCAT TNATNTCCTN
701  CCNNCCACTT TATGTGNCCA CCCCCANCNA CCNCCTTATN CCNGCCTTTC
751  TTCCTTANAA TTCCCCTTCC CCCTCTCTNT CCTCNTCTNT CTCTACTNCA
801  TNCTNGTTCC ACCTTNCCTC CTGGACTTTT NCTCCTCTTN TNTCCTCCCC
851  NATGTTCCCT TTCCNNTCAN TTTCNACNCT NTCTTGNTTT TNNCTCTCC
```

Figure 3.31. 12C (Cp1) genomic DNA sequence, length 899 nucleotides long.

```
  1  NCCCCCCCCT TTCCCCCCCT NTTCCTTATT TTAGGCGNCN TTTAAAAGGG
 51  CCCGTNCTGG GCCCCTTGTT GAAATNGCGG NTTTCGGAAT TCNACTNTAC
101  CGGTGGATTC NCCCNNGTNC TACGANNTCT TCTAACTCGC TCTTNGGGNT
151  NACCTGCNNT TANNTTNAGG NCTTCTTCNC NCANGCCTNT NTNGCCTCAT
201  TCNNNCTATN CCTTCCNTCC NGATCTTTNC TCNCTNNNNA NCTCCNNTCT
251  CCTANNTNTN CCACCTCCTC NGGCTCNTAN TCCTGNTCTN CNGNGACGTN
301  GCTCTTNTTC ATNCCTCTTC TNCCCNNCNN ATCCTCTTCC NTCATTCNTT
351  CCCCTCTCGN NTNGCNTTCC NTTTTNTTTT TTTTNNCNNG TCCNCCNCTN
401  NCCCCCTCTC NCCNCTCCCC TGACACATNT TTCTATTTTT CGCNGCTCNC
451  NCAGNTATCN NNTTTGGTTT TCTTNCTTTT CTNCTCCCNT NCCCTTCCTN
501  TNCTNNTNGG TNTNTTTCTT TNTCTNCNTC TCNCCNTCNA CTTTNCNCGT
551  CCCCCAANTT NNTCTTCCTT CCCCCCTTCT ACNTCCNAGC GTACTCCTTC
601  TCCCCTCTCC NGCCTCTCNN TCTCTTGTTT TNCTNNTTCT TTTTCCCTCC
651  TATTTTCNGC ATCTCCTCCT NNTTTCCTCT CTCTTCNTAN ATCTNTTCCC
701  TCCTTNCTNC CCTCGTTTAC NNANCNNCCC ACCNTTTCCT CACCCCATCC
751  NTTTTNNCCN TTNCTCNCTT NCCNAGCTCN TTCACNCTCN GGTCTCCTNT
801  TNNNCCCTTC TNCTTNTGTC NNNCTNNNNT GCNTCTCGCC TTTCTCNCCT
851  CCTTCTNNNT CCNCCCNCCN TCNCNCCTTT CTTTCCCTCN CTTCCTTNGC
901  TGTNTCCCNN NCCTCTCAGT TCTCNTCNCT NTTTCCTTCC NTTCNNC
```

Figure 3.32. 12C (Cr4) cDNA sequence, length 947 nucleotides long.

Initial alignments of the three sequences to the *A. bisporus* hspA gene sequence using the Wisconsin GCG gap comparison program (www.seqnet.dl.ac.uk) resulted in 85.9 % similarity of the control Mp2 sequence (Figure 3.33) demonstrating that the experimental rationale behind the primer design was vindicated to cloning and sequencing. Comparison of *A. bisporus* (Mp2) control sequence to Bb24 genomic DNA (Bp4), 12C genomic DNA (Cp1) and 12C cDNA (Cr4) sequences resulted in a low % similarity, indicating the gene sequences cloned for both *Serpula* species did not resemble the *A. bisporus* hspA gene sequence (Figure 3.34 A - C). Areas highlighted in blue refer to the longest regions of alignment with highest sequence homology. Table 2.2.8 summarises the results obtained from the GCG gap comparisons against *A. bisporus* hspA, partial gene sequence.

Generated unknown fungal sequences	GCG gap comparison to *A. bisporus* hspA gene sequence	
	% Similarity	% Identity
Mp2	85.9	79.8
Bp4	49.7	36.9
Cp1	40.4	33.4
Cr4	49.7	30.2

Table 3.2.8. GCG gap comparison of unknown gene sequences, Mp2, Bp4, Cp1 and Cr4 to *A. bisporus* hspA partial sequence (Accession no. X98505).

Figure 3.33. GCG Gap comparison of Mp2 nucleotide sequence aligned to *A. bisporus* partial sequence of hspA (Accession number X98508).

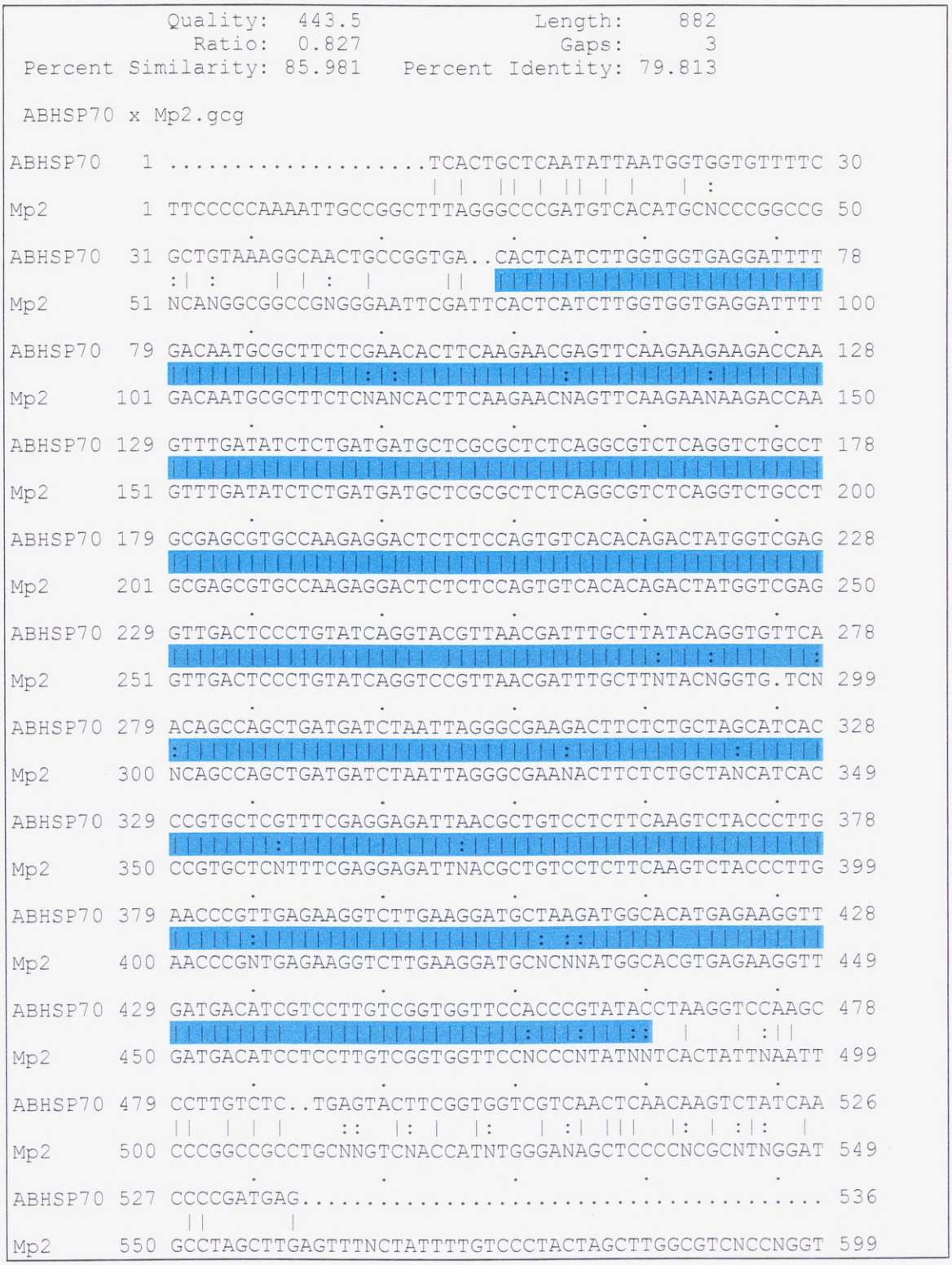


Figure 3.34 A. GCG Gap comparison of Bp4 nucleotide sequence aligned to *A. bisporus* partial sequence of hspA (Accession number X98508).

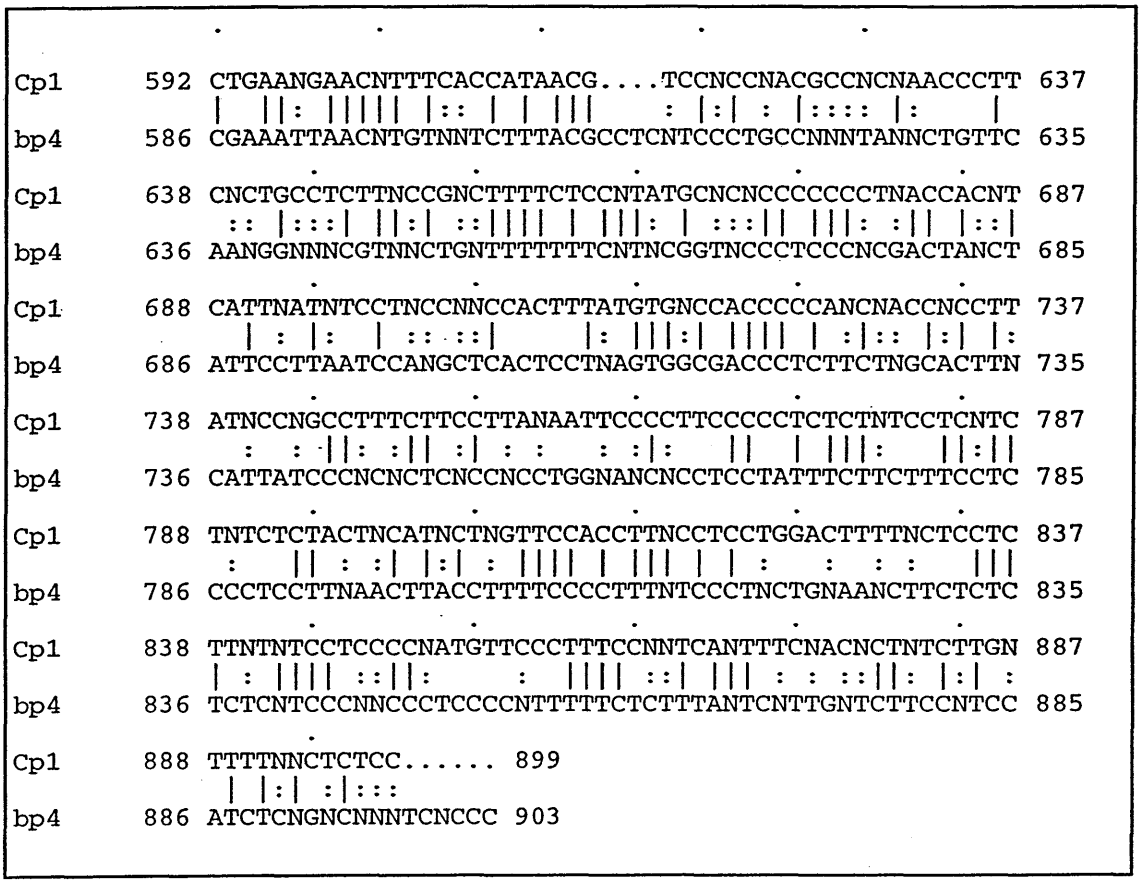
```
        Gap Weight:  5.000      Average Match:  1.000
     Length Weight:  0.300   Average Mismatch:  0.000

           Quality:  224.4             Length:    743
             Ratio:  0.419               Gaps:      6
Percent Similarity: 49.718   Percent Identity: 36.911

x98508 x bp4.gcg

      1 .................................TCACTGCTCAATATTA 16
                                        |   |||  |   | |
    163 GCAGGTCGACCATATGGGANAGCTCCCAACGCGTTGGATGCATAGCTTGA 212
                .         .         .         .         .
     17 ATGGTGGTGTTTTCGCTGTAAAGGCAACTGCCGGTGACACTCATCTTGGT 66
        |  |       |   :  ||||   | ||    || |   |  || | |
    213 GTATTCTATAGTGTCNCCTAAA..TAGCTTGGCGTAATCATGGTCATAGC 260
                .         .         .         .         .
     67 GGTGAGGATTTTGACAATGCGCTTCTCGAACACTTCAAGAACGAGTTCAA 116
        ||    : | ||| | ||   | | |  ||| ||| | | :   | ::|
    261 TGTTTCCNGTGTGAAATTGTTATCCGCTCACAATTCCACACNACATANNA 310
                .         .         .         .         .
    117 GAAGAAGACCAAGTTTGATA............TCTCTGATGATGCTCGCG 154
        |  | | :|  |  :|| :|               :|||: :  ||  |
    311 GCCGGAANCATAAANTGTNAAGCCTGGGGTGCCTANTGANTNATCTAACT 360
                .         .         .         .         .
    155 CTCTCAGGCGTCTCAGGTCTGCCTGCGAGCGTGCCAAGAGGACTCTCTCC 204
        | |  : :   |   |  ||  |||:  || | |||:  ||      |:
    361 CACATNANTTGCGTTGCGCTCACTGNCCGCTTTCCANTCGG.GAAACNGT 409
                .         .         .         .         .
    205 AGTGTCACACAGACTATGGTCGAGGTTGACTCCCTGTATCAGGTACGTTA 254
          |: ||:   :| ||: |     |  :|||||| |    |||   :||
    410 CCTNCCANCTGNATTANTGATTCTGCCNACTCCCGGGGAGAGGCGGNTTG 459
                .         .         .         .         .
    255 ACGATTTGCTTATACAGGTGTTCAACAGCCAGCTGATGATCTAATTAGGG 304
           :|| | :  |     : |||  | :|  ||:||  :||  :   ||
    460 CGTNTTGGGNGCTCTTCCNCTTC..CCNCTCACTNATTCNCTGCNCTCGG 507
                .         .         .         .         .
    305 CGAAGACTTCTCTGCTAGCATCACCCGTGCTCGTTTCGAGGAGATTAACG 354
        : :     | :: ||   :    : | : | | ||| ::|  |  |:||
    508 NCNTTCGGTTNNCGCGTANCGGTNTCCNCCCCCTTTANNGCGGTATNACT 557
                .         .         .         .         .
    355 CTGTCCTCTTCAAGTCTACCCTTGAACCCGTTGAGAAGGTCTTG....AA 400
         | : |   :|||  ||    ||   ||||     || :| |::     |
    558 GTTNTCCACNCAATCCTGGGATTTCCCCCGAAATTAACNTGTNNTCTTTA 607
                .         .         .         .         .
    401 GGATGCTAAGATGGCACATGAGAAGGTTGATGACATCGTCCTTG..... 444
         |   |:    || |:::| ::  | |  |:| :::|||:: ||
    608 CGCCTCNTCCCTGCCNNNTANNCTGTTCAANGGNNNCGTNNCTGNTTTTT 657
                .         .         .         .         .
    445 .....TCGGTGGTTCCACCCGTATACCTAAGGTCCAAGCCCTTGTCTCTG 489
           :||||:    | |:||  ||:|||      || || :  || ||
    658 TTCNTNCGGTNCCCTCCCNCGACTANCTATTCCTTAATCCANGCTCACTC 707
                .         .         .         .         .
    490 AGTACTTCGGTGGTCGTCAACTCAACAAGTCTATCAACCCCGATGAG... 536
          :| |   |       |   : ||   :   |||| :|:|  :  :
    708 CTNAGTGGCGACCCTCTTCTNGCACTTNCATTATCCCNCNCTCNCCNCCT 757
```

Figure 3.34 B. GCG Gap comparison of Cp1 nucleotide sequence aligned to *A. bisporus* partial sequence of hspA (Accession number X98508).

```
        Gap Weight:  5.000      Average Match:  1.000
     Length Weight:  0.300   Average Mismatch:  0.000

           Quality:  185.0             Length:    546
             Ratio:  0.370               Gaps:      2
Percent Similarity: 40.408   Percent Identity: 33.469

x98508 x Cp1.gcg

      1 .........TCACTGCTCAATATTAATGGTGGTGTTTTCGCTGTAAAGGC 41
                |  :    || |      | | : :    || | |::
      1 NCCCCCCCTTTTNCCACCATTTGCCCGGCTTNANGGCCCGATTTNNCATG 50
                .         .         .         .         .
     42 AACTGCCGGTGACACTCATCTTGGTGGTGAGGATTTTGACAATGCGCTTC 91
        :|       :| :  |  | : | |      |||| |      ||
     51 CNCCCGGCCGNAANGGCGGCCNTGGGAATTCGATTATACGGGTGGAACCA 100
                .         .         .         .         .
     92 TCGAACACTTCAAGAACGAGTTC.AAGAAGAAGACCAAGTTTGATATCTC 140
         ||| |||     ||||  || |  |  |  | :   |||      |
    101 CCGACCACCCTGGGAACTGGTCCTCACCACCAANATGAGTGAATCACTAA 150
                .         .         .         .         .
    141 TGATGATGCTCGCGCTCTCAGGCGTCTCAGGTCTGCCTGCGAGCGTGCCA 190
        |||    ||   |||   ||||     ||  |  | : ||   |    |
    151 TGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGANAGCTCCCAACGCG 200
                .         .         .         .         .
    191 AGAGGACTCTCTCCAGTGTCACACAGACTATGGTCGAGGTTGACTCCCTG 240
           |     |  |  | ||     | |:| |   | |: | |  |  |
    201 TTGGATGCATAGCTTGAGTATTCTATANTGTCACCTANATAGCTTGGCGT 250
                .         .         .         .         .
    241 TATCAGGTACGTTAACGATTTGCTTATACAGGTGTTCAACAGCCAGCTGA 290
         |||| |  | |     | :|    | |  |  ||||   |  :||
    251 AATCATGGTCATAGCTGTNTCCTGTGTGAAATTGTTATCCGCNCA..... 295
                .         .         .         .         .
    291 TGATCTAATTAGGGCGAAGACTTCTCTGCTAGCATCACCCGTGCTCGTTT 340
           | | |      |:|:    :  | |  ||||| | : ||:     |
    296 ....CAATTCCACACNANATACNAGCCGGAAGCATAAANTGTNAAGCCTG 341
                .         .         .         .         .
    341 CGAGGAGATTAACGCTGTCCTCTTCAAGTCTACCCTTGAACCCGTTGAGA 390
        :|  |  : |   |    || : |||  |  |         |:|     |
    342 NGGTGCCNATTGAGTGAGCCANCTCACATTAATTGCGTTGCNCTCACTGC 391
                .         .         .         .         .
    391 AGGTCTTGAAGGATGCTAAGATGGCACATGAGAAGGTTGATGACATCGTC 440
          |  ||  |:  :|  ||  :| |:    ||  | :| |    |||  |
    392 CCGCTTTCCANTCNGGAAACCNGTCNTGCCAGCTGCNTTAATGAATCTGC 441
                .         .         .         .         .
    441 CTTGTCGGTGGTTCCACCCGTATACCTAAGGTCCAAGCCCTTGTCTCTGA 490
        |    ||  || | ||  ||  |  |  |::   :  | |  :||
    442 CAACGCGCGGGGTACAGGCGGTTTGCGTANNGGGCNCTCTTCCNCTTCCT 491
                .         .         .
    491 GTACTTCGGTGGTCGTCAACTCAACAAGTCTATCAACCCCGATGAG 536
              : |
    492 CGCTCANTG..................................... 500
```

Figure 3.34 C. GCG Gap comparison of Cr4 nucleotide sequence aligned to *A. bisporus* partial sequence of hspA (Accession number X98508).

```
        Gap Weight:  5.000      Average Match:  1.000
     Length Weight:  0.300   Average Mismatch:  0.000

           Quality:  249.3             Length:    956
             Ratio:  0.465              Gaps:       2
Percent Similarity: 49.715   Percent Identity: 30.171

x98508 x cr4.gcg

      1 ..................TCACTGCTCAATATTAATGGTGGTGTTTTCGC 32
                               :|| ||  : :  :    : :|:   ||
    151 NACCTGCNNTTANNTTNAGGNCTTCTTCNCNCANGCCTNTNTNGCCTCAT 200
                .         .         .         .         .
     33 TGTAAAGGCAACTGCCGGTGACACTCATCTTGGTGGTGAGGATTTTGACA 82
        | :::    : || ||:   : |    |: | :  :::: : |  :: |
    201 TCNNNCTATNCCTTCCNTCCNGATCTTTNCTCNCTNNNNANCTCCNNTCT 250
                .         .         .         .         .
     83 ATGCGCTTCTCGAACACTTCAAGAACGAGTTCAAGAAGAAGACCAAGTTT 132
          ::|: :| | | | ||: |  |:  :||  |:   : : : |  |:
    251 CCTANNTNTNCCACCTCCTCNGGCTCNTANTCCTGNTCTNCNGNGACGTN 300
                .         .         .         .         .
    133 GATATCTCTGATGATGCTCGCGCTCTCAGGCGTCTCAGGTCTGCCTGCGA 182
        | | | : | ||:   ||   :| |:: ::   |||    :|   | :
    301 GCTCTTNTTCATNCCTCTTCTNCCCNNCNNATCCTCTTCCNTCATTCNTT 350
                .         .         .         .         .
    183 GCGTGCCAAGAGGACTCTCTCCAGTGTCACACAGACTATGGTCGAGGTTG 232
         |   |   :: : |: || :   |:|        :: ::|  |:  : |:
    351 CCCCTCTCGNNTNGCNTTCCNTTTTNTTTTTTTTNNCNNGTCCNCCNCTN 400
                .         .         .         .         .
    233 ACTCCCTGTATCAGGTACGTTAACGATTTGCTTATACAGGTGTTCAACAG 282
        :| |||| | :| : | |  | ||   |   :| |  |  | |   :
    401 NCCCCCTCTCNCCNCTCCCCTGACACAT...NTTTCTATTTTTCGCNGCT 447
                .         .         .         .         .
    283 CCAGCTGATGATCTAATTAGGGCGAAGACTTCTCTGCTAGCATCACCCGT 332
        |: :| |:|   ::: || |        :||| |||:||  |:|: ||
    448 CNCNCAGNTATCNNNTTTGGTTTTCTTNCTTTTCTNCTCCCNTNCCCTTC 497
                .         .         .         .         .
    333 GCTCGTTTCGAGGAGATTAACGCTGTCCTCTTCAAGTCTACCCTTGAACC 382
          : : |:: :|| : :|  |  |:|| :|:||  : |: |:  |  :|:
    498 CTNTNCTNNTNGGTNTNTTTCTTTNTCTNCNTCTCNCCNTCNACTTTNCN 547
                .         .         .         .         .
    383 CGTTGAGAAGGTCTTGAAGGATGCTAAGATGGCACATGAGAAGGTTGATG 432
        |||      | :| ::       | |      |   ||:|  :|  ||
    548 CGTCCCCCAANTTNNTCTTCCTTCCCCCCTTCTACNTCCNAGCGT..... 592
                .         .         .         .         .
    433 ACATCGTCCTTGTCGGTGGTTCCACCCGTATACCTAAGGTCCAAGCCCTT 482
          || | ||   |  |  : || | |::   | |    |:| ::  |||
    593 .ACTCCTTCTCCCCTCTCCNGCCTCTCNNTCTCTTGTTTTNCTNNTTCTT 641
                .         .         .         .         .
    483 GTCTCTGAGTACTTCGGTGGTCGTCAACTCAACAAGTCTATCAACCCCGA 532
        |  |    || ||  :   ||  |  ||::     ||| ||  |:  :|
    642 TTTCCCTCCTATTTTCNGCATCTCCTCCTNNTTTCCTCTCTCTTCNTANA 691
                .         .         .         .
    533 TGAG.......................................... 536
        |  :
    692 TCTNTTCCCTCCTTNCTNCCCTCGTTTACNNANCNNCCCACCNTTTCCTC 741
```


Cp1 592 CTGAANGAACNTTTCACCATAACG....TCCNCCNACGCCNCNAACCCTT 637
bp4 586 CGAAATTAACNTGTNNTCTTTACGCCTCNTCCCTGCCNNNTANNCTGTTC 635
Cp1 638 CNCTGCCTCTTNCCGNCTTTTCTCCNTATGCNCNCCCCCCCTNACCACNT 687
bp4 636 AANGGNNNCGTNNCTGNTTTTTTTCNTNCGGTNCCCTCCCNCGACTANCT 685
Cp1 688 CATTNATNTCCTNCCNNCCACTTTATGTGNCCACCCCCANCNACCNCCTT 737
bp4 686 ATTCCTTAATCCANGCTCACTCCTNAGTGGCGACCCTCTTCTNGCACTTN 735
Cp1 738 ATNCCNGCCTTTCTTCCTTANAATTCCCCTTCCCCCTCTCTNTCCTCNTC 787
bp4 736 CATTATCCCNCNCTCNCCNCCTGGNANCNCCTCCTATTTCTTCTTTCCTC 785
Cp1 788 TNTCTCTACTNCATNCTNGTTCCACCTTNCCTCCTGGACTTTTNCTCCTC 837
bp4 786 CCCTCCTTNAACTTACCTTTTCCCCTTTNTCCCTNCTGNAANCTTCTCTC 835
Cp1 838 TTNTNTCCTCCCCNATGTTCCCTTTCCNNTCANTTTCNACNCTNTCTTGN 887
bp4 836 TCTCNTCCCNNCCCTCCCCNTTTTTCTCTTTANTCNTTGNTCTTCCNTCC 885
Cp1 888 TTTTNNCTCTCC..... 899
bp4 886 ATCTCNGNCNNNTCNCCC 903

Further analysis using the FastA and GCG search engines (www.seqnet.dl.ac.uk), the sequences of 12C and Bb24 were initially compared against all the gene sequences identified in the EMBL: fungal database. Further searches were undertaken using a variety of gene databases, the results for which are individually summarised in the following sections. All nucleotide sequences were truncated prior to analysis to exclude regions possibly relating to the vector sequence. The sequences were trimmed at a position corresponding to 101 and 850 bp. The remaining conserved "core" sequences between 101 and 850 bp were then used for the FastA searches.

The FastA uses the method of Pearson and Lipman (1988) to search for similarities between the query sequence (unknown) and any marked group of sequences (EMBL: fungal). In the first step of this search, the comparison can be viewed as a set of dot plots, with the query as the vertical sequence and the group of sequences to which the query is being compared as the different horizontal sequences. This first step finds the registers of comparison (diagonals) having the largest number of short perfect base-pair matches (or words) for each comparison. In the second step, these "best" regions are re-scored using a scoring matrix that allows conservative replacements, ambiguity symbols, and runs of identities shorter than the size of the word. In the third step, the program checks to see if some of these initial highest-scoring diagonals can be joined together. Finally, the searches set sequences with the highest scores are aligned to the query sequence for display.

A FastA search was initiated to compare Cp1, Bp4 and Cr4 nucleotide sequences to the EMBL: Fungal gene database. The primary search summarised in Tables 3.2.9 and 3.3.0, show the single pass sequencing of the putative clones of *Serpula* species to have homology to a number of fungal sequences.

Name and Description	Accession number	Percentage Homology	
		Cp1 Percentage Homology in a Nucleotide Overlap	Bp4 Percentage Homology in a Nucleotide Overlap
S. pombe (clone pJK210) DNA	L25928	74% identity in 562 nt overlap	66.8% identity in 723 nt overlap
S. pombe (clone pJK148) DNA	L25927	74% identity in 562 nt overlap	66.8% identity in 723 nt overlap
S. cerevisiae CUP1	U37457	76% identity in 495 nt overlap	67.7% identity in 661 nt overlap
Candida sp. CIP1 gene	Y13973	89% identity in 275 nt overlap	84.8% identity in 270 nt overlap
S. pombe rAD17 checkpoint gene	X91889	84% identity in 231 nt overlap	79% identity in 240 nt overlap
Pichia pastoris	Z46234	66% identity in 236 nt overlap	58% identity in 311 nt overlap
Pichia pastoris	Z46232	66.9% identity in 236 nt overlap	58% identity in 311 nt overlap
Pichia pastoris	Z46233	66.9% identity in 236 nt overlap	58.5% identity in 311 nt overlap
Pichia pastoris	Z46231	66.9% identity in 236 nt overlap	58.5% identity in 311 nt overlap
S. cerevisiae BIO3 and BIO4 genes	U53467	91% identity in 105 nt overlap	92% identity in 105 nt overlap

Table 3.2.9. Comparison of sequence homologies from FastA EMBL: fungal database search to Cp1 and Bp4.

Name and Description	Accession number	Cr4 Percentage Homology in a Nucleotide Overlap
S. cerevisiae XI chromosome	Z28201	47.9% identity in 471 nt overlap
S. cerevisiae DNA from XV chromosome	X90565	54.7% identity in 201 nt overlap
S. cerevisiae XV chromosome	Z75217	54.7% identity in 201 nt overlap
A. niger zinc finger protein	L03811	50% identity in 330 nt overlap
N. crassa tryptophan synthetase	J04594	56.2% identity in 219 nt overlap
S. cerevisiae chromosome XVI	Z71255	52.9% identity in 238 nt overlap
S. cerevisiae chromosome	Z71255	52.9% identity in 238 nt overlap
S. cerevisiae chromosome	U33335	52.9% identity in 238 nt overlap
S. cerevisiae chromosome XVI cosmid	Z48483	52.9% identity in 238 nt overlap

Table 3.3.0. Comparison of sequence homologies from FastA EMBL: fungal database search to Cr4.

The highest percentage identity for both Cp1 and Bp4 was against the *S. pombe* (clone pJK210) DNA sequence accession number L25928 (Keeney and Boeke, 1994), with the longest nucleotide overlap of 562 and 732 respectively (Table 3.2.9). In comparison there was no apparent high homology for the 12C cDNA clone, Cr4 against any of the highlighted EMBL: Fungal sequences (Table 3.3.0). The FastA search of the EMBL: fungal database also illustrated the difference's between the genomic (Cp1 and Bp4) and cDNA (Cr4) generated sequences as the searches were completely different in each case. The alignment of Bp4 and Cp1 sequences were further compared by GCG gap comparison to *S. pombe* (Accession no. L25928), illustrated in Figure 3.35 A and B.

Figure 3.35 A. GCG gap comparison illustrates the sequence similarity of Bp4 to *S. pombe* gene sequence (SPSEQB).

```
           Quality:  500.0              Length:    829
             Ratio:  0.678                Gaps:     10
Percent Similarity: 76.164   Percent Identity: 64.795

spseqb x bp4.gcg

  2670 ....GAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCG 2715
                | ||   :  ||     |   ||| ||||||  |    ||
   113 GCGCCCTCACCACCAANATGAGTGAATCACTATTGAATTCGCGGCCGCCT 162
                .         .         .         .         .
  2716 GGGGATCCACTAGTTCTAGAGCGGCCGCCACCGCGGTGGAGCTCCAGCTT 2765
       |  | || || |  | | |  : ||  ||| |||| ||||     |||||
   163 GCAGGTCGACCA..TATGGGANAGCTCCCAACGCGTTGGATGCATAGCTT 210
                .         .         .         .         .
  2766 TTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTTGGCGTAATCATGGTCAT 2815
         ||   || |||||  :  ||| |    ||||||||||||||||||||
   211 GAGTATTCTATAGTGTCNCCTAAAT...AGCTTGGCGTAATCATGGTCAT 257
                .         .         .         .         .
  2816 AGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATA 2865
       ||||||||||:|||||||||||||||||||||||||||||||:|||||
   258 AGCTGTTTCCNGTGTGAAATTGTTATCCGCTCACAATTCCACACNACATA 307
                .         .         .         .         .
  2866 GGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGGTA 2915
       ::|||||||||:|||||||:|||:|||||||||||||||||:|||:|:|  ||
   308 NNAGCCGGAANCATAAANTGTNAAGCCTGGGGTGCCTANTGANTNATCTA 357
                .         .         .         .         .
  2916 ACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACC 2965
       ||||||||:|:|||||||||||||||||:||||||||||:|||||||| |
   358 ACTCACATNANTTGCGTTGCGCTCACTGNCCGCTTTCCANTCGGGAAA.C 406
                .         .         .         .         .
  2966 TGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGT 3015
       :||| |:|||:|||:||||:||| || |||:|| | |||||||||||||:
   407 NGTCCTNCCANCTGNATTANTGATTCTGCCNACTCCCGGGGAGAGGCGGN 456
                .         .         .         .         .
  3016 TTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTC 3065
       ||||||:|||||:|||||||||:|||||  |:|||||||:|  ||:||||:|||
   457 TTGCGTNTTGGGNGCTCTTCCNCTTCC.CNCTCACTNATTCNCTGCNCTC 505
                .         .         .         .         .
  3066 GGTCGTTCGGCTGCGGCG.AGCGGTATCAGCTCACTCAAAGGCGGTAATA 3114
       ||:|:|||||  |:: ||| |:||||:|| :|  | ||  |::|||||| :|
   506 GGNCNTTCGGTTNNCGCGTANCGGTNTCCNCCCCCTTTANNGCGGTATNA 555
                .         .         .         .         .
  3115 CGGTTATCCACAGAATCAGGGGATAACGCAGGAAA.GAACATGTGAGCAA 3163
       | |||:||||||: ||||  |||||  | |  ||||  |||:|||:: |
   556 CTGTTNTCCACNCAATCCTGGGATTTCCCCCGAAATTAACNTGTNNTCTT 605
                .         .         .         .         .
  3164 AAGGCCAGCAAAAGGCCAGG..AACCGTAAAAAGGCCGCGTTGCTGGCGT 3211
         | |||  :      |||:::  ::| ||  ||:||:::|||::|| |: |
   606 TACGCCTCNTCCCTGCCNNNTANNCTGTTCAANGGNNNCGTNNCT.GNTT 654
                .         .         .         .         .
  3212 TTTTCCATAGGCTCGGCCCCCCTGACGAGCATCACAAAAATCGACGCTCA 3261
       |||| |:|: | |:  | |||: ||| |:|    |   |||| |:||||
   655 TTTTTCNTNCGGTNCCCTCCCNCGACTANCTATTCCTTAATCCANGCTC. 703
                .         .         .         .         .
```

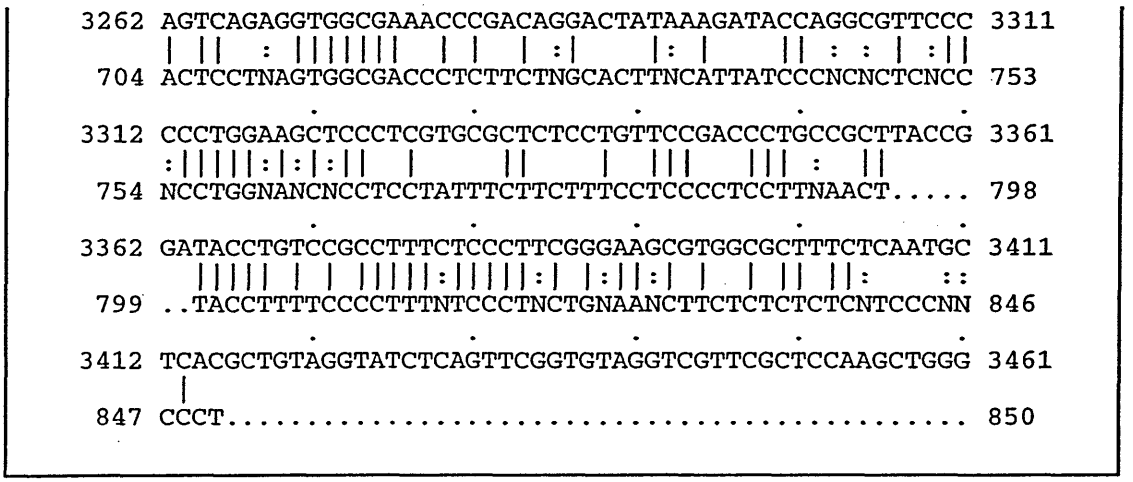
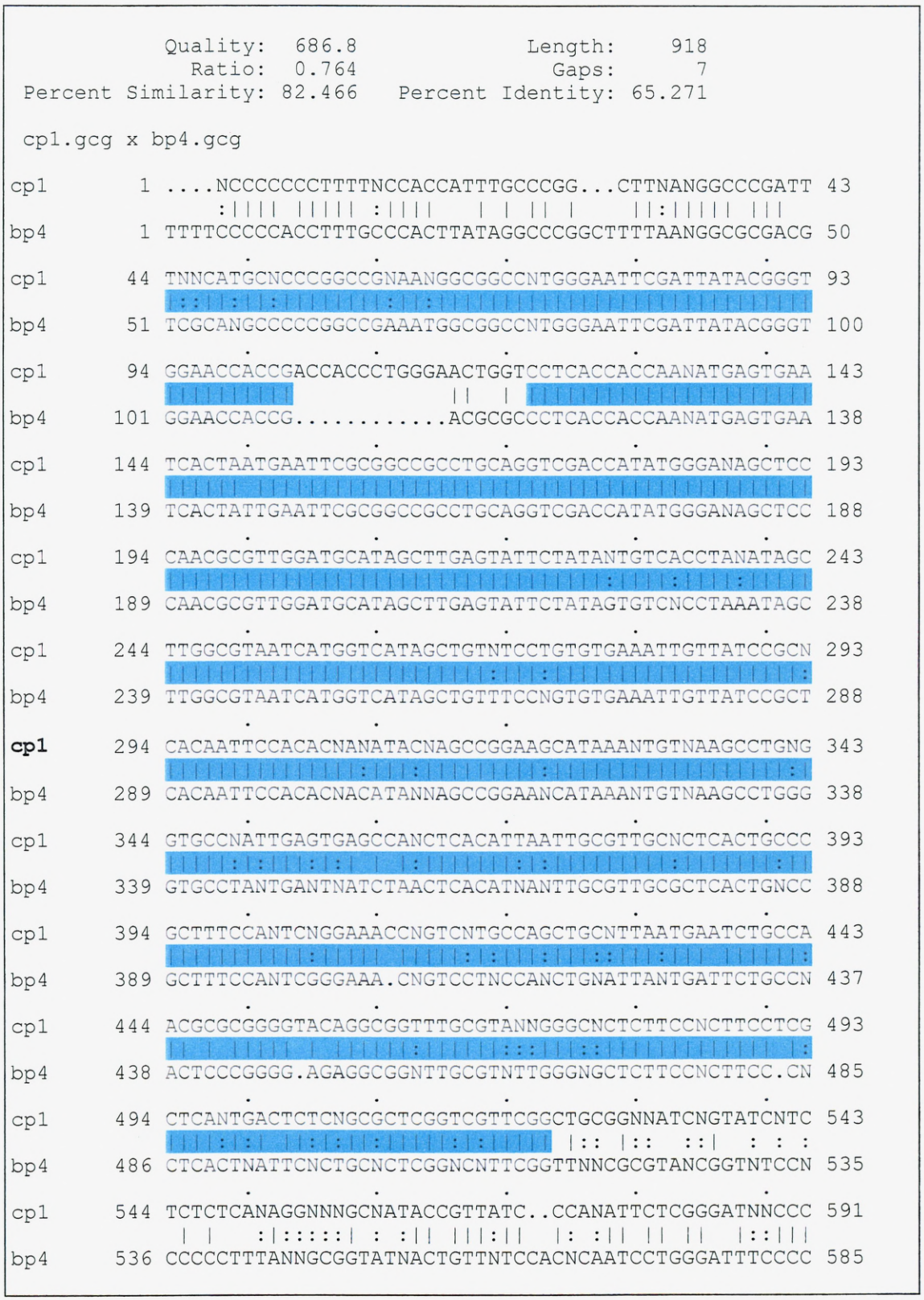

3262 AGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTCCC 3311
704 ACTCCTNAGTGGCGACCCTCTTCTNGCACTTNCATTATCCCNCNCTCNCC 753
3312 CCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCG 3361
754 NCCTGGNANCNCCTCCTATTTCTTCTTTCCTCCCCTCCTTNAACT..... 798
3362 GATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGC 3411
799 ..TACCTTTTCCCCTTTNTCCCTNCTGNAANCTTCTCTCTCTCNTCCCNN 846
3412 TCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGG 3461
847 CCCT..... 850

Figure 3.35 B. GCG gap comparison illustrates the sequence similarity of Cp1 to *S. pombe* gene sequence (SPSEQB).

```
        Gap Weight:  5.000      Average Match:  1.000
     Length Weight:  0.300   Average Mismatch:  0.000

           Quality:  295.7             Length:    943
             Ratio:  0.591               Gaps:      4
Percent Similarity: 84.392   Percent Identity: 77.778

spseqb x cp1.gcg


  2670 ..............GAGGTCGACGGTATCGATAAGCTTGA.....TAT 2698
                          ||||  |   | | |:| |  |||     ||
   101 CCGACCACCCTGGGAACTGGTCCTCACCACCAANATGAGTGAATCACTAA 150
                .         .         .         .         .
  2699 CGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGCCACCG 2748
       ||||||  |    || |  | || || |  | | |  : ||  ||| ||
   151 TGAATTCGCGGCCGCCTGCAGGTCGACCA..TATGGGANAGCTCCCAACG 198
                .         .         .         .         .
  2749 CGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTGCGCGCTT 2798
       || ||||     |||||  ||   || ||:||     ||: |    ||||
   199 CGTTGGATGCATAGCTTGAGTATTCTATANTGTCACCTANAT...AGCTT 245
                .         .         .         .         .
  2799 GGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCA 2848
       ||||||||||||||||||||||||:||||||||||||||||||||||:||
   246 GGCGTAATCATGGTCATAGCTGTNTCCTGTGTGAAATTGTTATCCGCNCA 295
                .         .         .         .         .
  2849 CAATTCCACACAACATAGGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGT 2898
       ||||||||||:|:||| :|||||||||||||||:|||:|||||||:|||
   296 CAATTCCACACNANATACNAGCCGGAAGCATAAANTGTNAAGCCTGNGGT 345
                .         .         .         .         .
  2899 GCCTAATGAGTGAGGTAACTCACATTAATTGCGTTGCGCTCACTGCCCGC 2948
       |||:| ||||||||  |:||||||||||||||||||:||||||||||||
   346 GCCNATTGAGTGAGCCANCTCACATTAATTGCGTTGCNCTCACTGCCCGC 395
                .         .         .         .         .
  2949 TTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAAC 2998
       ||||||:||:||||||||:|||:||||||||||:|||||||||| ||||||
   396 TTTCCANTCNGGAAACCNGTCNTGCCAGCTGCNTTAATGAATCTGCCAAC 445
                .         .         .         .         .
  2999 GCGCGGGG.AGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCT 3047
       |||||||| | ||||||||||||||::||||:||||||||:||||||||||
   446 GCGCGGGGTACAGGCGGTTTGCGTANNGGGCNCTCTTCCNCTTCCTCGCT 495
                .         .         .         .         .
  3048 CACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTC 3097
       ||:||
   496 CANTG..................................................  500
```

Due to the high homology of both Cp1 and Bp4 to *S. pombe* (Accession no. L25928), the question as to whether the two sequences are related was investigated. GCG gap comparison of the two gene sequences, resulted in an 82 % similarity, demonstrating that the sequences are closely related (Figure 3.36). This was further established with alignment of 44 - 523 (Cp1) and 51 - 515 (Bp4) nucleotides resulting in an almost continuous homology between this region (highlighted in blue, Figure 3.36).

Figure 3.36. GCG Gap comparison of Cp1 and Bp4 aligned nucleotide sequences.



There are distinct similarities between Cp1 and Bp4 sequence lengths and positions in relation to their alignment to the EMBL sequences generated from the FastA search. Table 3.3.1 illustrates where core regions within both sequences are highly homologous to the EMBL test sequences. These core regions of high homology are clearly highlighted in blue.

		Position and Lengths of Core Regions with Highest Sequence Similarity					Position and Lengths of Core Regions with Highest Sequence Similarity				
Name and Description	**Accession number**	**EMBL Position**	**Length**	**12C Position**	**No. of bp Matches**	**% Similarity**	**EMBL Position**	**Length**	**Bb24 Position**	**No. of bp Matches**	**% Similarity**
S. pombe (clone pJK210) DNA	L25928	2795..3081	287	242..529	258	89.8	2794..3075	282	235..517	242	85.8
S. pombe (clone pJK148) DNA	L25927	3106..3392	287	242..529	258	89.8	3104..3384	281	235..517	241	85.7
S. cerevisiae CUP1	U37457	3326..3612	287	242..529	258	89.8	3327..3605	282	240..521	243	86.0
Candida sp. CIP1 gene	Y13973	1037..1310	274	255..529	245	89.0	1034..1300	270	245..513	230	85.0
S. pombe rAD17 gene	X91889	5282..5480	223	244..470	195	87.0	5277..5441	165	260..423	145	87.8
Pichia pastoris	Z46234	5926..5981	56	474..529	51	91.0	5926..5968	61	467..527	48	78.7
Pichia pastoris	Z46232	5913..5968	56	474..529	51	91.0	5915..5976	61	474..526	47	77.0
Pichia pastoris	Z46233	4673..4728	56	474..529	51	91.0	4676..4736	61	467..527	47	77.0
Pichia pastoris	Z46231	4660..4715	56	474. 529	51	91.0	4663..4723	61	464..526	47	77.0
S. cerevisiae BIO3 and BIO4 genes	U53467	4398..4502	105	241..345	96	91.0	4395..4498	105	235..340	97	92.0

Table 3.3.1. Position and lengths of core regions found in Cp1 and Bp4 with high homology to fungal database sequences.

Due to this high homology of core regions found in each of the EMBL: fungal sequences, a further search of the STS database was initiated to eliminate the possibility that the sequences for Bp4 and Cp1 were derived from the actual vector DNA (refer to Appendix K, K7). Table 3.3.2 summarises the results STS search. It clearly illustrates a high homology of Cp1 and Bp4 sequences to a number of plasmid cloning vectors with the occurrence of core regions of homology (Table 3.3.3). The highest similarity of 74 % in a 673 nt overlap and 72 % in a 699 nt overlap to Bp4 and Cp1 respectively were to the following: pGEM-5zf (+), accession number - X65309; pGEM-5zf (-) accession number - X65311 and pSG928, accession number - U14121. It became apparent the sequences cloned for the *Serpula* species could in fact be sections of the plasmid vector, pGEM-T.

This might also explain why there was a high percentage similarity to the *S. pombe* (Accession no. L25928) gene sequence as the region of highest homology corresponds to the region which is homologous to the plasmid vector illustrating that the cloned *S. pombe* product by Keeney and Boeke, (1994) has part of the plasmid vector sequenced.

Name and Description	Accession number	Percentage Homology	
		Cp1 Percentage Homology in a Nucleotide Overlap	Bp4 Percentage Homology in a Nucleotide Overlap
pGEM-5zf(+) vector	X65308	74 % identity in a 673 nucleotide overlap	72 % identity in a 699 nucleotide overlap
pGEM-5zf(-) vector	X65309	74 % identity in a 673 nucleotide overlap	72 % identity in a 699 nucleotide overlap
pSG928 cloning vector	U14121	74 % identity in a 673 nucleotide overlap	72 % identity in a 699 nucleotide overlap
pGEM-7zf(-) vector	X65311	73 % identity in a 647 nucleotide overlap	71 % identity in a 673 nucleotide overlap
pGEM-7zf(+) vector	X65310	73 % identity in a 647 nucleotide overlap	71 % identity in a 673 nucleotide overlap
Ligation independent vector	U25268	73 % identity in a 647 nucleotide overlap	71 % identity in a 673 nucleotide overlap
Ligation independent vector	U25272	73 % identity in a 647 nucleotide overlap	71 % identity in a 673 nucleotide overlap
pSG929 vector	U14119	73 % identity in a 647 nucleotide overlap	71 % identity in a 673 nucleotide overlap
pLVH-1 vector	AF041426	71 % identity in a 688 nucleotide overlap	69 % identity in a 712 nucleotide overlap
pSG930 vector	U14118	70 % identity in a 708 nucleotide overlap	68 % identity in a 734 nucleotide overlap

Table 3.3.2. Comparison of sequence homologies from FastA STS Genbank search to Cp1 and Bp4.

		Position and Lengths of Core Regions with Highest Sequence Similarity					Position and Lengths of Core Regions with Highest Sequence Similarity				
Name and Description	**Accession number**	**EMBL Position**	**Length**	**Cp1 Position**	**No. of bp Matches**	**% Similarity**	**EMBL Position**	**Length**	**Bp4 Position**	**No. of bp Matches**	**% Similarity**
pGEM-5zf(+) vector	X65308	61..431	371	158..529	341	91.9	61..425	365	153..515	324	88.7
pGEM-5zf(-) vector	X65309	61..431	371	158..529	341	91.9	61..425	365	153..515	324	88.7
pSG928 cloning vector	U14121	713..1083	371	157..528	340	91.0	714..1078	365	153..515	324	88.7
pGEM-7zf(-)	X65311	84..429	345	184..529	315	91.0	84..422	339	179..515	298	87.9
pGEM-7zf(+)	X65310	84..429	345	184..529	315	91.0	48..422	339	178..515	298	87.9
Ligation independent vector	U25268	117..461	345	184..529	315	91.0	117..455	339	179..515	297	87.6
Ligation independent vector	U25272	117..461	345	184..529	315	91.0	117..455	339	179..515	303	89.4
pSG929	U14119	737..1081	345	184..529	315	91.0	737..1075	339	179..515	255	75.0
pLVH-1	AF041 426	4916..5251	336	193..529	306	91.0	4912..5245	333	184..515	291	87.0
pSG930 vector	U14118	759..1083	325	204..529	296	91.0	759..1077	319	199..515	281	88.0

Table 3.3.3. Position and lengths of core regions found in Cp1 and Bp4 with high homology to STS database sequences.

3.5.5 Summary and Concluding Remarks.

The expression of specific hsp70 mRNA was analysed by RT-PCR followed by cloning and sequencing of the reaction products for identification. Successful identification of the *A. bisporus* control was achieved, but the sequences generated from the reaction products of Bb24 and 12C were not conclusively identified as part of an hsp70 gene sequence.

The use of PCR for the analysis of RNA's is not as commonly used as DNA amplification, partly due to other methods of RNA analysis can be quantified, while PCR in this instance can only provide information on the presence or absence of a given sequence. Quantitative PCR has limitations, partly due to the final PCR product is derived from the exponential amplification of the starting template, hence minor differences in amplification efficiencies can result in large differences in product yield, especially if the starting template is low. A linear relationship between starting template and PCR product within the exponential range of amplification can be observed. This relationship is dependent on sample preparation, thermal cycler performance and inhibitors, which is especially critical in RT-PCR. To overcome sample - to sample variation use of internal controls have been developed (Arnheim and Erlich, 1992; Bouaboula et. al. 1992; Delidow et. al. 1989; Gilliland et. al. 1990), such as the 'house-keeping' gene, GAPDH that allows comparison and normalisation of expression levels (Baier et. al. 1993). Semi-quantification can be achieved by further modifications of the procedure: optimising DNase removal of contaminating DNA from RNA samples (Huang et. al. 1996), as contaminating DNA can produce incorrect results because of its potential to act as a second competitor; modification of reaction conditions by optimising annealing temperatures, as this is a primer dependent step; varying dilution's of RNA produce proportional changes in the PCR-amplified product (Delidow et. al. 1989). This latter step was clearly seen when initial experiments were set out to determine the optimum dilution of *A.*

bisporus mushroom RNA treated and untreated with DNase, as shown in Figure 3.24. This problem can be eliminated in theory by treating samples with DNase, but precise reaction conditions are required. The most effective removal of this problem is again the crucial design of the primers. By choosing primers that span introns, the length of the amplified region differs for the genomic DNA and mRNA. This was clearly demonstrated when comparing the size of the genomic and cDNA products illustrated in Figure 3.27.

Through a systematic search of the Gene Databases, close comparisons were made to *S. pombe* (clone pJK210) DNA sequence, accession number - L25928 with a 66.8 % identity in 723 nt overlap and 74 % identity in 562 nt overlap for Bp4 and Cp1 respectively (FastA search results, Table 3.2.9). There was no clear indication to the identity of Cr4, 12C cDNA product as the database searches resulted in no high percentage similarities to any sequences (FastA results, Table 3.3.0). Further search of the STS database highlighted sequence similarities to a number of plasmid vectors: pGEM-5zf (+), accession number - X65309; pGEM-5zf(-) accession number - X65311 and pSG928, accession number - U14121 all having a 74 % similarity in a 673 nt overlap to Bp4 and 72 % in a 699 nt overlap to Cp1 respectively. The probability of having sequenced part of the pGEM-T vector rather that the inserted fragment is high and further cloning will have to be done to establish the identity of the amplimers generated from RT-PCR and PCR experiments.

3.4 The Detection and Identification of AMP-Activated Protein Kinase in *Serpula* Species.

The primary aim of this investigation was to detect immunologically the presence of a possible AMP-activated protein kinase (AMPK) fungal homologue in *Serpula* species, by the use of anti-AMPK polyclonal antiserum raised against the rat AMPK protein, isolated from liver extracts. Positive identification of an appropriate antigen would allow for activity to be investigated using an assay system developed by Dr. S. P. Davies at the Department of Biochemistry, University of Dundee. The polyclonal antiserum and respective control protein used in this study were also kindly donated by Dr. Davies.

Figure 3.37 A - D illustrates the detection of an AMPK homologue in the intracellular extracts of Bb24 only (Blot A). No apparent signal is seen for 12C, although a band running at approximately 41.7 kD is detected (Blot B and lanes 4 - 5 in Blot C). An antigenic signal (67 kD) is detected in all of the Bb24 extracts with the exception of the control (Train C) and the resultant recovery (lane 8 and 10 respectively). The antigenic signal also appears to be of a slightly higher molecular weight to the control protein (Blot D). It has been demonstrated that AMPK is expressed only during periods of stress (Refer to Introduction). This can be clearly demonstrated for Bb24 cultures exposed to Train C (control) treatment regimes. During stress free periods, stimulated by optimum conditions, no AMPK is detected (lane 8, Blot A). When cultures are exposed to extreme heat treatment (lane 9), a positive signal is detected which disappears when cultures are placed back into optimum conditions (lane10). This observation of an antigenic signal occurring in Bb24 but not 12C intracellular extracts is similar to that observed in the immunological studies (Section 3.2) to detect homologues of hsp60 and 70 *in*

vivo by polyclonal antibodies (Figures 3.15 B and 3.17 B) and MAb's (Figures 3.18 and 3.19 B).

AMPK can easily be used as a marker to identify periods of stress, in this case heat. The primary role AMPK has in eukaryotic cells under stress is to protect the ATP pools for crucial 'house-keeping' activities. This possibly allows the cell to survive these periods of stress.

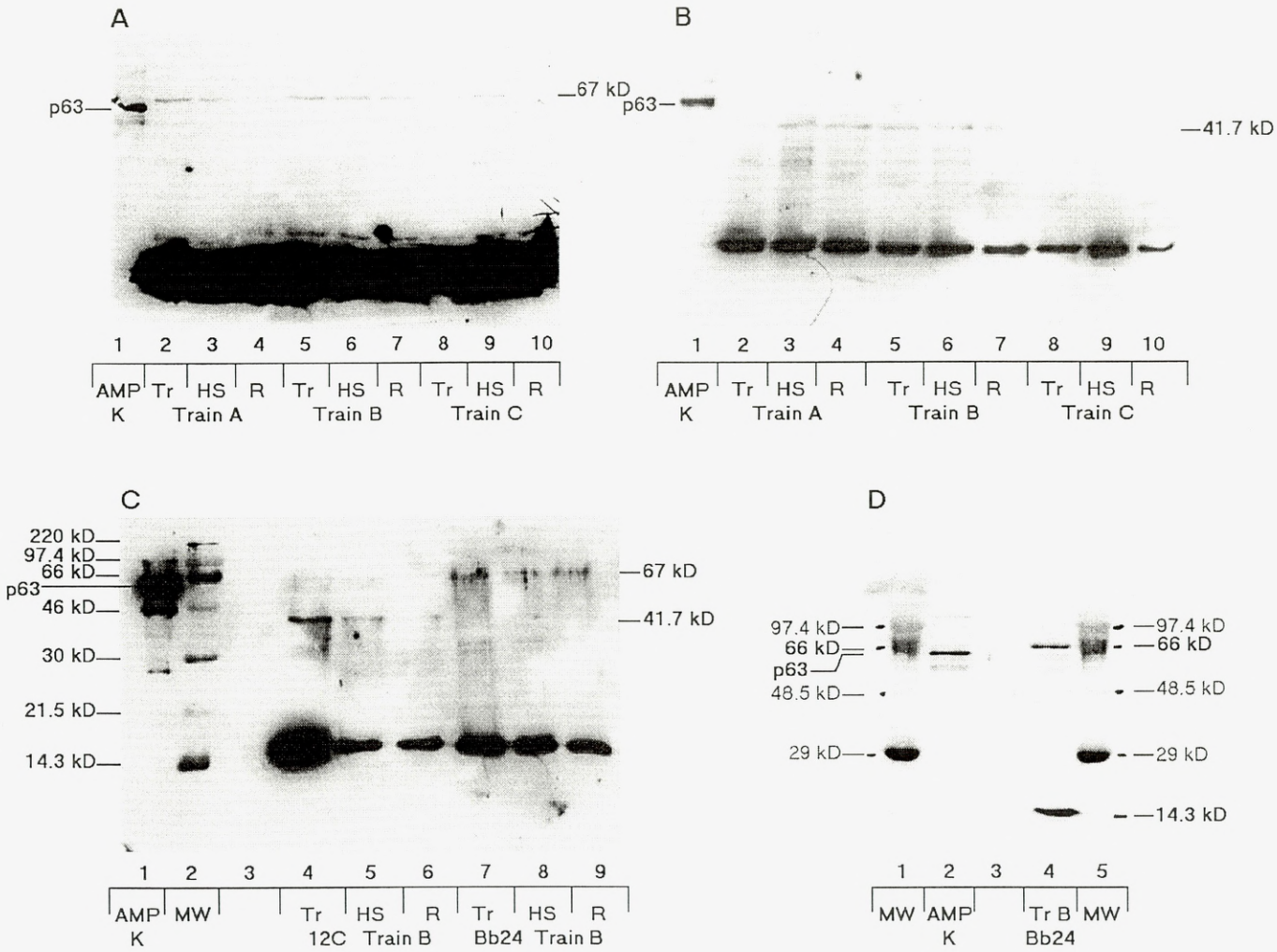


Figure 3.37 A - D. ECL Western blot analysis for the detection of AMPK in Bb24 and 12C intracellular extracts of cultures which have undergone various temperature regimes using 1:4000 dilution of anti-αPAN polyclonal antiserum. Samples were equalised for total protein content of 6 μg. Blot D was set out by Dr. S. P. Davies. Refer to Figure 3.15 A and B for legend information for (A) and (B).

Lane 1 on both blots (A and B) - 3.6 μg of AMP-K control protein (purified from rat liver).

(C) Lane 1 - AMPK, 3.6 μg of AMP-K control protein (purified from rat liver). Lane 2 - MW, rainbow high molecular weight markers in the range of 14.3 - 222 kD (Amersham International plc.). Lane 4 - 6 represent 12 Train B cultures lane 4 - Train B; lane 5 - extreme heat treatment; lane 6 - recovery. Lane 7 - 9 represent Bb24 Train B cultures: lane 7 -Train B; lane 8 - extreme heat treatment; lane 9 - recovery.

(D) Lane 1 and 5 - MW, high molecular weight standards range 29 - 97.4 kD (Sigma, cat. M5505). Lane 2 - AMPK, 3.6 μg of AMP-K control protein (purified from rat liver). Lane 4 - Bb24, Train B.

After the preliminary identification of cross-reactive material in the immunological study, the next question as to whether whole fungal cell extracts showed AMPK activity was investigated. The simple and sensitive assay developed by Dr. Davies utilised a synthetic peptide, which is based on the exact amino acid sequence around the unique site (Ser79), which is phosphorylated exclusively by AMP-activated protein kinase. Using a synthetic peptide substrate based on this sequence kinase activity was determined. No activity was measured in any of the intracellular extracts of Bb24 and 12C tested (data not shown), indicating that the enzyme was either denatured, inactive or levels of activity were too low to be detected.

3.5 Neutral Trehalase Activation and Trehalose Mobilisation During the Heat-Shock Response.

The remarkable property of the trehalose metabolising system is its constant readiness for an almost instantaneous adjustment of the trehalose level in eukaryotic cells. The link between increased levels of trehalose and elevated levels of stress tolerance or thermotolerance has been demonstrated in a number of eukaryotic studies (Attfield, 1987; Hottiger et. al., 1987a; De Virgilio et. al., 1990; Panek and Panek, 1990; Styrvold and Strom, 1991). The large amount of trehalose accumulated under these conditions have been shown to be rapidly degraded by neutral trehalase when the cells are shifted back into optimum temperature conditions (Thevelein, J. M., 1984c; De Virgilio et. al., 1991b).

The main aims of the experiments included in this section were:-

(i) To determine whether there is a direct correlation between elevated temperatures and increased levels of trehalose in cultures of *S. lacrymans* and *S. himantioides*.

(ii) To determine if a transient increase in trehalose is correlated with induced thermotolerance.

(iii) To investigate the possible role of neutral trehalase in trehalose metabolism during the heat-shock response, by monitoring the levels of trehalase activity during sublethal, extreme and recovery phases.

(iv) To investigate the relationship between trehalose accumulation during extreme heat treatment and neutral trehalase activity during the recovery period.

The following sub-sections outline the summary of results for the analysis of trehalose and its degradative enzyme trehalase in trained and untrained cultures of both 12C and Bb24.

3.5.1 The Comparative Analysis Trehalose Accumulation in Control and Trained 12C and Bb24 cultures.

Tables 3.3.4 A and B summarises the effects of sublethal training on cultures of Bb24 and 12C. There are distinct differences between the level of trehalose present in the two species. These are clearly illustrated in Figure 3.38.

(A)

Bb24 Training Conditions	Trehalose %Dry Weight
Train A, growth at 28 °C/ 48 hr	4.8 ± 0.8
Train B, growth at 30 °C/ 48 hr	6.2 ± 1.3
Train C, optimum growth at 25 °C/ 24 hr	1.4 ± 0.5

(B)

12C Training Conditions	Trehalose % Dry Weight
Train A, growth at 28 °C/24 hr	11.97 ± 1.61
Train B, growth at 25 °C/48 hr	5.18 ± 2.41
Train C, optimum growth at 22 °C/24 hr	6.73 ± 1.34

Table 3.3.4 A and **B.** Accumulation of trehalose in trained (Train A and B) and control (Train C) cultures of Bb24 and 12C respectively, as percentage of dry weight. Average of 5 replicate ± standard deviation.

Figure 3.38 illustrates the distinct differences between Bb24 and 12C with respect to trehalose accumulation. Overall the levels of trehalose for trained (Train A and B) and control (Train C) cultures are higher in 12C compared to Bb24. This is clearly demonstrated in control 12C cultures with approximately 4.8 times greater level of trehalose present than in Bb24 control cultures. Differences in the effect of sublethal training between the two species is also apparent with the greatest increase in 12C Train A cultures, percentage dry weight of 11.97 compared to the value of 4.8 for Bb24. This difference is not only reflected between the species but within the individual training regimes applied to each species. There is approximately 2.3 fold increase in the level of trehalose in 12C Train A to that of B cultures (Table 3.3.4 B). The opposite of this occurs in Bb24 cultures, with the highest value of 6.2 % and slightly less, 4.8 % for Train B and Train A respectively.

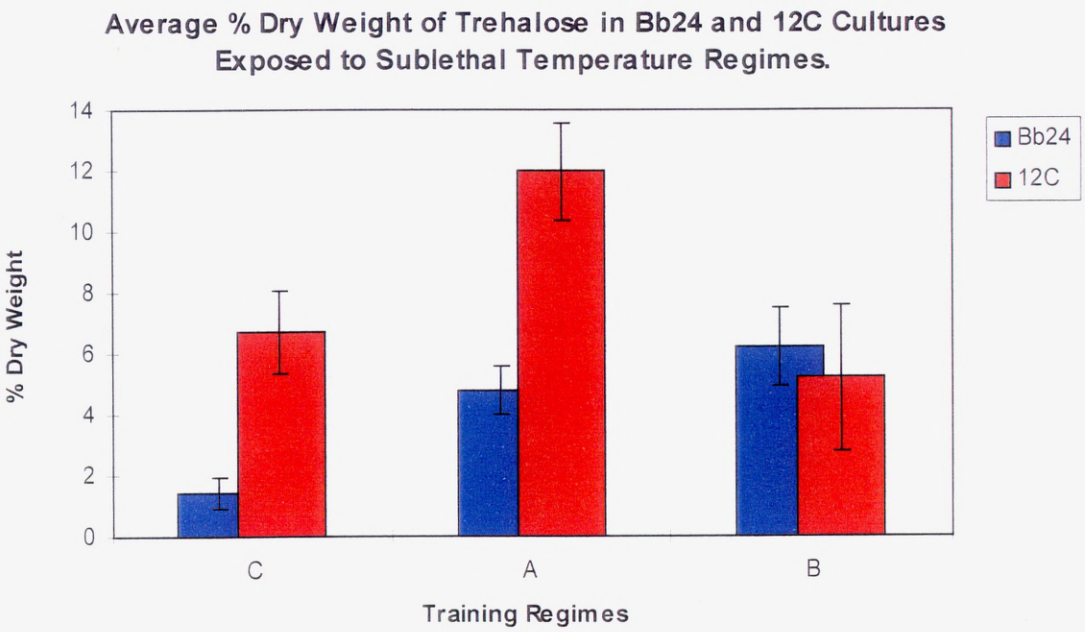


Figure 3.38 Accumulation of trehalose in Bb24 (■) and 12C (■) Trained (A and B) and control (C) cultures.

The following Sections 3.5.2 and 3.5.3 summarise the changes in trehalose accumulation during sublethal, extreme and recovery regimes in Bb24 and 12C cultures. This was achieved by sampling at appropriate intervals throughout the time course of the various temperature regimes imposed, thus enabling a systematic analysis of these changes.

3.5.2 The Detection and Analysis of Trehalose in Bb24 Cultures Subjected to Sublethal (Trained), Extreme and Optimal (Train C) Temperature Regimes.

Table 3.3.5 summarises the changes in trehalose present in Train A, B and control (Train C) cultures of Bb24 subjected to extreme heat treatment and recovery periods.

Temperature Regime	Time (hrs)	Trehalose % Dry Weight		
		Train A	Train B	Train C
Train	0	4.8 ± 0.8	6.2 ± 1.3	1.4 ± 0.5
Extreme	0.5	4.3 ± 0.2	7.4 ± 1.3	1.6 ± 0.6
	1	3.6 ± 0.3	7.7 ± 1.0	1.8 ± 0.5
	1.5	3.4 ± 0.4	7.2 ± 2.1	3.2 ± 1.3
	2	3.3 ± 0.5	7.2 ± 0.9	3.9 ± 2.0
	2.5	5.9 ± 0.4	6.2 ± 0.9	3.5 ± 0.8
	3	5 ± 0.6	4.6 ± 0.9	3.7 ± 0.8
Recovery	3.5	5.5 ± 0.3	4.3 ± 0.6	1.1 ± 0.2
	4	7.3 ± 1.3	4.0 ± 1.1	1.6 ± 0.7
	4.5	8.8 ± 1.4	5.5 ± 1.0	2.3 ± 1.1
	5	5.3 ± 2.0	4.9 ± 1.4	2.6 ± 0.7
	5.5	****	****	****
	6	****	****	3.1 ± 0.7
	23	3.8 ± 0.2	1.9 ± 1.1	****
	24	3.5 ± 0..5	2.2 ± 1.2	4.4 ± 1.8

Table 3.3.5. Trehalose accumulation in Bb24 trained (Train A and B) and control (Train C) cultures. Data represents the average of 5 replicate ± standard deviation. The time course of 0 to 24 hr relates to the designated sampling times during the various temperature regimes used: starting at 0 hr for the end of a sublethal training period; 0.5 - 3 hr for sampling during extreme heat treatment; 3.5 - 24 hr sampling in the recovery period.

Overall the comparative analysis of the changing levels of trehalose present in Train A, B and C cultures summarised in the above Table 3.3.5, demonstrates a number of points, which are clearly illustrated in Figure 3.39.

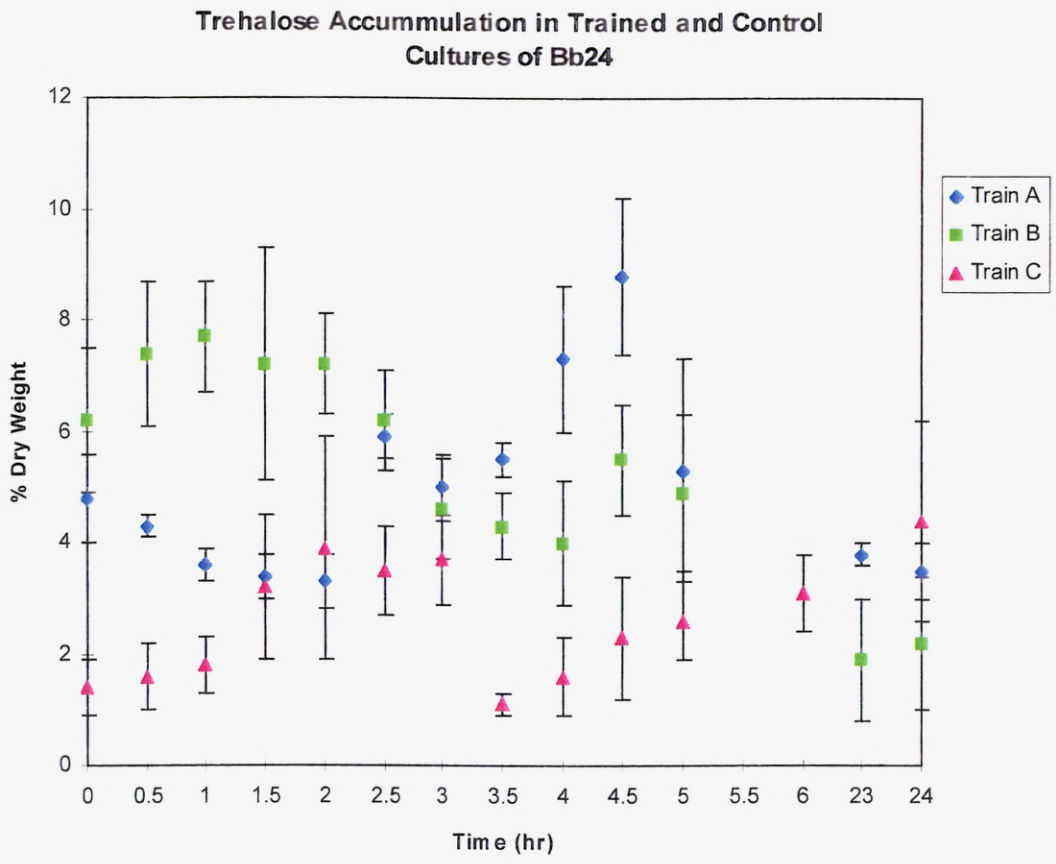


Figure 3.39. Percentage dry weights of trehalose for Bb24 Train A, B and C cultures which have undergone extreme heat treatment and recovery periods. Cultures (replicates of 5) were harvested every 30 mins during imposed temperature regimes. Train A: 0 hr, harvested cultures after exposure to 28 ^{0}C/ 48 hr; 0.5 - 2 hr period, Train A cultures harvested during exposure to extreme heat treatment (40 ^{0}C/ 3 hr); the period between 2.5 - 24 hr, cultures harvested during the recovery period (25 ^{0}C/ 24 hr). Train B: 0 hr, cultures harvested after exposure to 30 ^{0}C/ 48 hr; 0.5 - 2 hr period, Train B cultures harvested during exposure to extreme heat treatment (40 ^{0}C/ 3 hr); the period between 2.5 - 24 hr, cultures harvested during the recovery period (25 ^{0}C/ 24 hr). Train C: 0 hr, control cultures harvested after exposure to 25 ^{0}C/ 24 hr; 0.5 - 2 hr period, control cultures harvested during exposure to extreme heat treatment (40 ^{0}C/ 3 hr); the period between 2.5 - 24 hr, cultures harvested during the recovery period (25 ^{0}C/ 24 hr).

Firstly, there is increased levels of trehalose present in Train A and B sample between 0 - 1 hr compared to their control counterparts (Train C). Secondly, Train B, Bb24 cultures clearly demonstrate an overall increase in levels of trehalose during extreme heat treatment (0.5 - 3 hr), with the highest value at 1 hr of 7.7 %. There is a gradual decrease in levels of trehalose present in the first few hours of recovery (3.5 - 5 hr), with a slight increase occurring at 4.5 hr which again falls to a value of 2.2 % at the end of the recovery period (24 hr). Thirdly, Train C cultures demonstrate an overall increase in the levels of trehalose during exposure to extreme heat treatment, between 0.5 - 3 hr. After which the levels decrease dramatically from 3.7 to 1.1 % at the beginning of the recovery period (3.5 hr). After 1 hr in recovery (4 hr period, Figure 3.39), the levels start to gradually rise again to a maximum of 4.4 % at 24 hr. Lastly, Train A cultures reveal a different trend compared to those of Train B and C cultures. There is an increase in trehalose levels starting during extreme heat treatment, which continues through the recovery period and peaks at 4.5 hr with a value of 8.8 %, after which levels decrease to 3.5 % at 24 hr.

Comparison of trehalose levels between the respective Train A and B cultures, illustrates that incubating cultures at sublethal temperature of 30 ^{0}C for 48 hr (Train B) has the greatest effect on trehalose production during extreme heat treatment, with a decrease during recovery. But cultures incubated at 28 ^{0}C for 48 hr (Train A) exhibit trehalose production during recovery with a substantial increase in the first hour (3.5 - 4.5 hr periods, Figure 3.39).

3.5.3 The Detection and Analysis of Trehalose Accumulation in 12C Cultures Subjected to Sublethal (Train A and B), Extreme and Recovery Temperature Regimes.

12C cultures exposed to the various training, extreme heat treatment and recovery periods, were also monitored for changes in the levels of trehalose. Table 3.3.6 summarises the results obtained for 12C Train A, B and C cultures.

Temperature Regime	Time (hrs)	% Dry Weight of Trehalose		
		Train A	Train B	Train C
Train	0	11.97 ± 1.61	5.18 ± 2.41	6.73 ± 1.34
Lethal	0.5	8.72 ± 2.65	4.57 ± 1.01	6.62 ± 0.56
	1	14.56 ± 5.14	3.55 ± 0.69	6.85 ± 1.57
	1.5	14.95 ± 2.55	3.21 ± 2.41	8.27 ± 0.88
	2	13.88 ± 2.51	3.08 ± 0.59	9.61 ± 4.18
Recovery	2.5	14.16 ± 3.46	3.21 ± 0.60	8.24 ± 1.65
	3	12.11 ± 1.81	2.69 ± 0.40	10.63 ± 4.50
	3.5	12.76 ± 2.98	0.82 ± 0.27	7.49 ± 3.64
	4	12.92 ± 3.07	1.08 ± 0.70	7.04 ± 1.67
	4.5	14.50 ± 3.24	1.03 ± 0.62	7.10 ± 1.73
	5	13.02 ± 1.54	1.37 ± 1.02	****
	5.5	14.67 ± 1.7	****	****
	24	15.03 ± 2.5	2.01 ± 1.45	4.16 ± 1.93

Table 3.3.6. Trehalose accumulation in 12C trained (Train A and B) and control (Train C) cultures. Average of 5 replicate ± standard deviation. The time course of 0 to 24 hr relates to the designated sampling times during the various temperature regimes used: starting at 0 hr for the end of a sublethal training period; 0.5 - 2 hr for sampling during extreme heat treatment; 2.5 - 24 hr sampling in the recovery period.

The comparative analysis of the changing levels of trehalose present in Train A, B and C cultures summarised in the above Table 3.3.6, demonstrates a number of points, which are clearly illustrated in Figure 3.40.

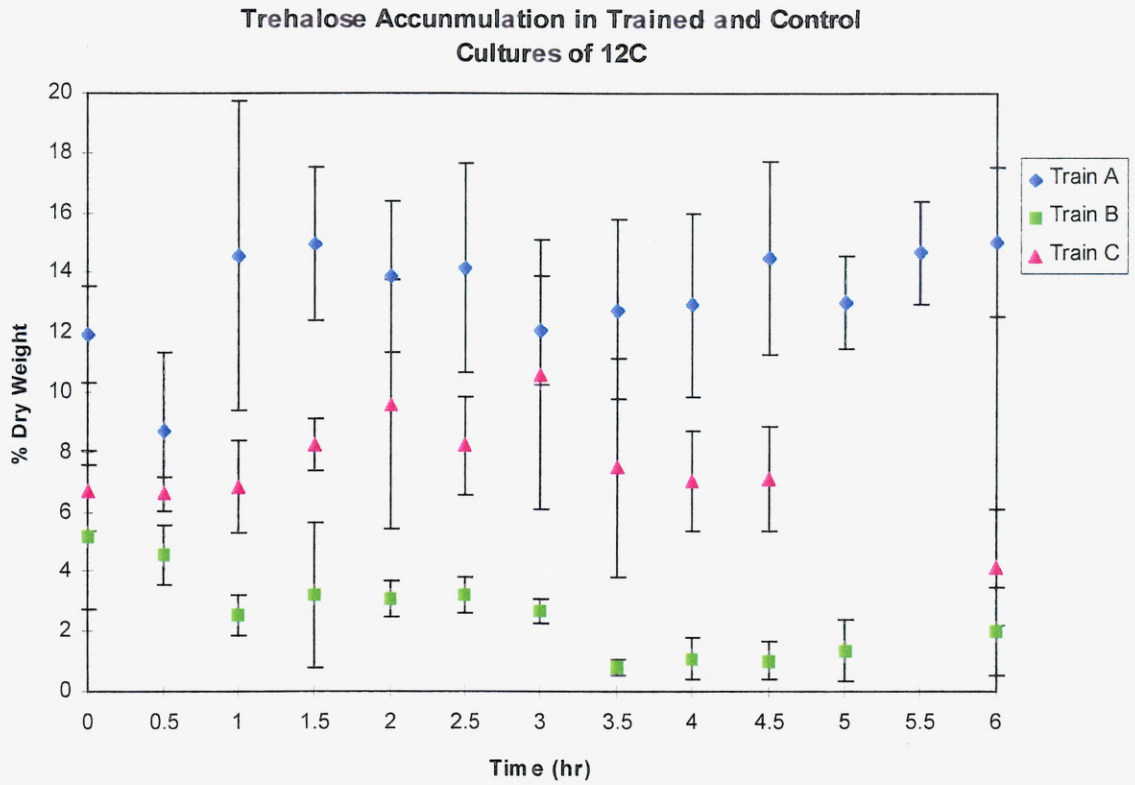


Figure 3.40. Percentages dry weight of trehalose in 12C Train A, B and C cultures which have undergone extreme heat treatment and recovery periods. Cultures (replicates of 5) were harvested every 30 mins during imposed temperature regimes. Train A: 0 hr represents Train A cultures harvested after exposure to 28 ^{0}C/ 24 hr; 0.5 - 2 hr period, Train A cultures harvested during exposure to extreme heat treatment (40 ^{0}C/ 2 hr); the period between 2.5 - 24 hr, cultures harvested during the recovery (22 ^{0}C/ 24 hr). Train B: 0 hr, Train B cultures harvested after exposure to 25 ^{0}C/ 48 hr; 0.5 - 2 hr period, Train B cultures harvested during exposure to extreme heat treatment (40 ^{0}C/ 2 hr); the period between 2.5 - 24 hr, cultures harvested during the recovery period (22 ^{0}C/ 24 hr). Train C: 0 hr, control cultures harvested after exposure to 22 ^{0}C/ 24 hr; 0.5 - 2 hr period, control cultures harvested during exposure to extreme heat treatment (40 ^{0}C/ 2 hr); the period between 2.5 - 24 hr, cultures harvested during the recovery period (22 ^{0}C/ 24 hr).

As with Bb24 there are a number of distinct features, which are illustrated, in the above Figure 3.40. There is a gradual decline in trehalose levels for Train B cultures, during the extreme heat treatment (0.5 - 2 hr). A slight increase is detected once cultures were placed into recovery conditions (3.21 % occurs at 2.5 hr). This is followed by a further decrease during the recovery to 1.03 % at 4.5 hr, with a possible increase appearing at the tail end of recovery to a value of 2.01 % at 24 hr.

Train A cultures produce the highest level of trehalose, starting at 11.97 % at 0 hr, with a sharp decline occurring in the first 30 min of extreme treatment (8.72 % at 0.5 hr). After this point a sharp increase occurs, which peaks at 14.95 % at 1.5 hr, followed by a slight fall at 2 hr. During the recovery period of 2.5 - 24 hr, high levels of trehalose are maintained 3.5 - 5 hr with minor fluctuations in between. After 5 hr, levels start to rise again, with the highest percentage recorded for 12C cultures at 24 hr in recovery of 15.03 %. As with Train cultures, the control cultures (Train C) show a similar trend where there are no distinct changes in trehalose levels with shifts in temperature. There is a gradual increase in levels starting in the first 30 min of extreme heat treatment (0.5 hr) and continuing through into recovery to the highest percentage of 10.63 at 3 hr. After 3hr period, levels gradually decrease to 4.16 % at the end of recovery (24 hr).

Comparisons between the Train A, B and C cultures, clearly shows that Train A cultures incubated at sublethal temperatures of 28 ^{0}C for 24 hrs showed the greatest change in the amount of trehalose produced during the subsequent extreme heat treatment and recovery periods. This is clearly demonstrated with the highest values ranging from 11.97 to 15.03 %.

The overall range of trehalose levels present for each time course of 12C Train A, B and control (Train C) cultures are higher compared to those of Bb24 counterparts (Figure 3.39).

This was clearly demonstrated when comparing the overall spread of values for Bb24, which is within the range of 1.4 - 8.8 % to that of 12C, with 1.03 - 15.03 %.

3.5.4 The Comparative Analysis of Specific Trehalase Activity Trained (Train A and B) and Control (Train C) 12C and Bb24 Cultures.

Tables 3.3.7 A and B summarises the effects of sublethal training on the specific trehalase activity in cultures Bb24 and 12C. There are distinct differences in the levels of activity in the two species and these are clearly illustrated in Figure 3.41.

(A)

Bb24 Training Conditions	Specific Trehalase Activity (10^6 U x µg protein^{-1})
Train A, growth at 28°C/ 48 hrs	0.892 ± 0.05
Train B, growth at 30°C/ 48 hrs	1.515 ± 0.297
Train C, growth at 25°C/ 24 hrs	3.139 ± 1.01

(B)

12C Training Conditions	Specific Trehalase Activity (10^6 U x µg protein^{-1})
Train A, growth at 28°C/ 24 hrs	0.784 ± 0.017
Train B, growth at 25°C/ 48 hrs	2.037 ± 0.124
Train C, growth at 22°C/ 24 hrs	1.237 ± 0.058

Table 3.3.7 A and **B.** Specific trehalase activity in trained and control (Train C) Bb24 and 12C cultures respectively. Average of 5 replicate ± standard deviation. Specific trehalase activity was determined whereby 1 Unit of trehalase activity is defined as the amount of enzyme which gives rise to 1 nmol of glucose/ min/ µg protein.

Figure 3.42 depicts the differences in trehalase activity between Bb24 and 12C. Comparison of Bb24 Train A, B and control cultures shows the most marked difference between the levels of trehalase activity measured in the respective sublethal temperature regimes. The highest level of specific trehalase activity is detected in Bb24 control (Train C) cultures with approximately 3.1 x 10^6 U/μg protein, which is approximately 3 times greater than that measured in 12C control counterparts (Table 3.3.7 B). The highest level of activity in 12C is seen for Train B cultures. While Train A cultures for both species have the lowest level of activity.

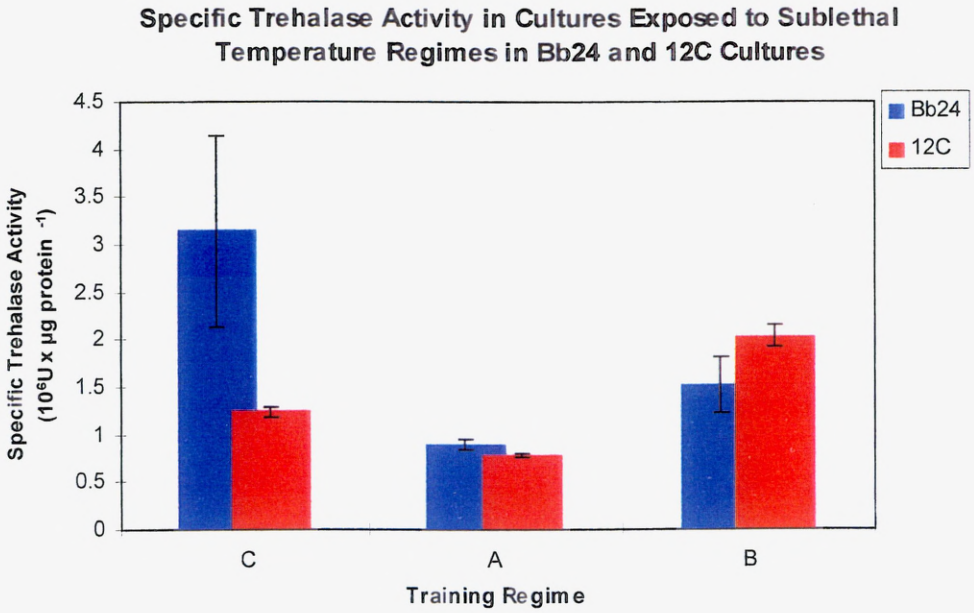


Figure 3.42. Specific trehalase activity in trained (Train A and B) and control (Train C) cultures of 12( )and Bb24 ( ).

The following Sections 3.5.5 and 3.5.6 summarise the changes in specific trehalase activity during sublethal, extreme and recovery regimes in Bb24 and 12C cultures. This was achieved by sampling at appropriate intervals throughout the time course of the various temperature regimes imposed.

3.5.5 The Detection and Analysis of Trehalase in Bb24 Cultures Subjected to Sublethal (Trained), Extreme and Recovery Temperature Regimes.

Table 3.3.8 summarises the changes in specific trehalase activity in Train A, B and control (Train C) cultures of Bb24 subjected to extreme heat treatment and recovery periods.

Temperature Regime	Time (hrs)	Specific Trehalase Activity (10^6 U x µg protein^{-1})		
		Train A[1]	Train B	Train C
Train	0	0.892 ± 0.05	1.515 ± 0.297	3.139 ± 1.01
Extreme	0.5	0.855 ± 0.063	1.451 ± 0.155	1.864 ± 1.587
	1	0.889 ± 0.075	1.764 ± 0.254	1.637 ± 0.657
	1.5	1.073 ± 0.07	1.68 ± 0.176	0.985 ± 0.512
	2	0.917 ± 0.068	1.891 ± 0.414	1.966 ± 1.257
	2.5	0.917 ± 0.072	1.712 ± 0.194	1.789 ± 0.399
	3	0.882 ± 0.065	1.64 ± 0.102	1.973 ± 0.360
Recovery	3.5	0.876 ± 0.062	1.932 ± 0.502	1.737 ± 0.316
	4	0.877 ± 0.077	2.957 ± 0.509	1.651 ± 0.328
	4.5	0.897 ± 0.069	2.735 ± 0.219	1.742 ± 0.200
	5	0.979 ± 0.025	2.676 ± 0.368	1.293 ± 0.247
	5.5	****	****	1.193 ± 0.159
	6	****	****	1.337 ± 0.094
	23	0.945 ± 0.057	2.917 ± 0.205	****
	24	0.952 ± 0.151	2.604 ± 0.21	1.484 ± 0.215

Table 3.3.8. Specific trehalase activities of Bb24 trained and control (Train C) cultures. Average of 5 replicate ± standard deviation, Train A samples prefixed with ([1]) represents replicates of 3. The time course of 0 to 24 hr relates to the designated sampling times during the various temperature regimes used: starting at 0 hr for the end of a sublethal training regime; 0.5 - 3 hr for sampling during extreme heat treatment; 3.5 - 24 hr sampling in the recovery period. Specific trehalase activity was determined whereby 1 Unit of trehalase activity is defined as the amount of enzyme which gives rise to 1 nmol of glucose/ min/ µg protein.

The comparative analysis of changes in trehalase activity present in Train A, B and C cultures summarised in Table 3.3.8, demonstrates a number of points illustrated in Figure 3.43.

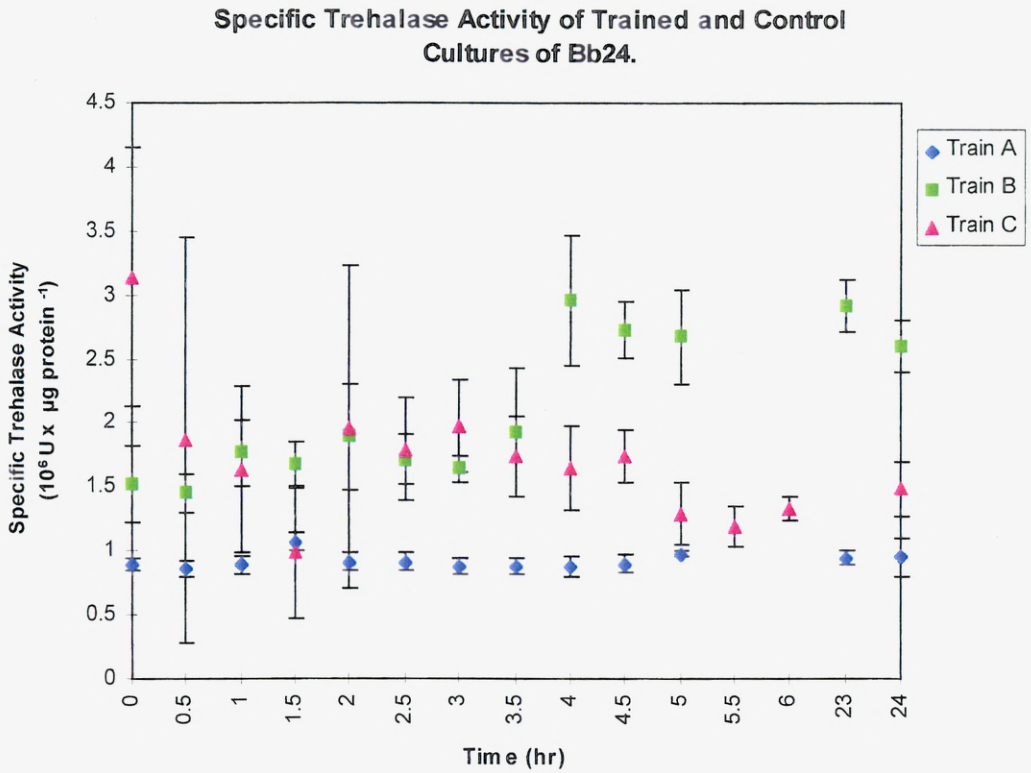


Figure 3.43. Specific trehalase activity of Bb24 Train A, B and C cultures which have undergone extreme heat treatment and recovery periods. Cultures were harvested every 30 min and specific trehalase activity was determined whereby 1 Unit of trehalase activity is defined as the amount of enzyme which gives rise to 1 nmol of glucose/ min/ µg protein. Train A: 0 hr represents cultures harvested after training at sublethal temperatures of 28 ^{0}C/ 48 hr; 0.5 - 2 hr period, Train A cultures harvested during exposure to extreme heat treatment (40 ^{0}C/ 3 hr); the period between 2.5 - 24 hr, cultures are placed into recovery (25 ^{0}C/ 24 hr). Train B: 0 hr, cultures harvested after training at sublethal temperatures of 30 ^{0}C/ 48 hr; 0.5 - 2 hr period, Train B cultures harvested during exposure to extreme heat treatment (40 ^{0}C/ 3 hr); the period between 2.5 - 24 hr, cultures harvested during the recovery period (25 ^{0}C/ 24 hr). Train C: 0 hr, control cultures harvested after incubation at optimal conditions of 25 ^{0}C/ 24 hr; 0.5 - 2 hr period, control cultures harvested during exposure to extreme heat treatment (40 ^{0}C/ 3 hr); the period between 2.5 - 24 hr, cultures harvested during the recovery period (25 ^{0}C/ 24 hr).

Examination of the changes in trehalase activity in Bb24 cultures resulting from incubation at sublethal temperature (Train A and B) and the changes in the control (Train C) regimes, upon exposure to extreme heat and subsequent recovery periods shows distinct differences. Trehalase activity in extracts prepared from Train C (control) cultures at 0 hr produced the highest level of activity overall. Upon extreme heat treatment, the activity decreases to approximately half the value in the first 0.5 hr period. The period between 2 - 4.5 hr shows trehalase activity levelling out at around 1.7 x 10^6U/µg protein. After 4.5 hr, there is a continued decline in activity to a value of 1.484 x 10^6U/µg protein at 24 hr.

Exposing Bb24 cultures to sublethal temperature regime (Train A) appears to have no major effect on the level of trehalase activity throughout extreme and recovery periods. This is clearly demonstrated in Figure 3.43, with an almost constant level of activity present. It is also clear that the overall activity in Train A culture is less than that found in the control counterparts.

Values presented in Train B cultures demonstrates a trend whereby there is low activity during sublethal and extreme heat treatment regimes (0 - 3 hr), and an increase in activity upon a shift back into optimal conditions (3.5 - 24 hr). The greatest level of activity during the recovery phase is also demonstrated for Train B cultures with 2.957 x 10^6U/µg protein which is an approximate 3 and 1.8 fold increase in comparison to the respective Train A and C recovery counterparts.

3.5.6 The Detection and Analysis of Trehalase in 12C Cultures Subjected to Sublethal (Trained), Extreme and Recovery Temperature Regimes.

Changes in specific trehalase activity were monitored during the various temperature regimes applied to 12C cultures with the results summarised in Table 3.3.9.

Temperature Regime	Time (hrs)	Specific Trehalase Activity (10^6 U x µg protein^{-1})		
		Train A[1]	Train B	Train C
Train	0	0.784 ± 0.017	2.037 ± 0.124	1.237 ± 0.058
Extreme	0.5	0.83 ± 0.028	2.034 ± 0.059	1.187 ± 0.102
	1	0.86 ± 0.007	2.471 ± 0.162	1.218 ± 0.049
	1.5	0.856 ± 0.082	2.063 ± 0.151	1.238 ± 0.128
	2	0.855 ± 0.047	1.938 ± 0.283	1.156 ± 0.07
Recovery	2.5	0.876 ± 0.061	2.018 ± 0.423	1.156 ± 0.08
	3	0.909 ± 0.071	2.188 ± 0.731	1.177 ± 0.081
	3.5	0.915 ± 0.04	2.229 ± 0.39	1.088 ± 0.078
	4	0.854 ± 0.074	2.247 ± 0.368	1.222 ± 0.149
	4.5	1.177 ± 0.056	2.185 ± 0.121	1.236 ± 0.146
	5	1.392 ± 0.093	2.258 ± 0.366	****
	5.5	1.257 ± 0.08	****	****
	6	****	****	****
	23	****	****	****
	24	1.22 ± 0.064	2.082 ± 0.298	1.448 ± 0.06

Table 3.3.9. Specific trehalase activities of 12C trained and control (Train C) cultures. Average of 5 replicate ± standard deviation, Train A sample prefixed with ([1]) represent replicates of 3. The time course of 0 to 24 hr relates to the designated sampling times during the various temperature regimes used: starting at 0 hr for the end of a sublethal training regime; 0.5 - 2 hr for sampling during extreme heat treatment; 2.5 - 24 hr sampling in the recovery period. Specific trehalase activity was determined whereby 1 Unit of trehalase activity is defined as the amount of enzyme which gives rise to 1 nmol of glucose/ min/ µg protein.

Comparing the trehalase activity present in extracts of Train A, B and C cultures of 12C through shifts in temperature of extreme heat treatment and recovery periods illustrates similar trends to those found for Bb24 cultures which is clearly illustrated in Figure 3.44.

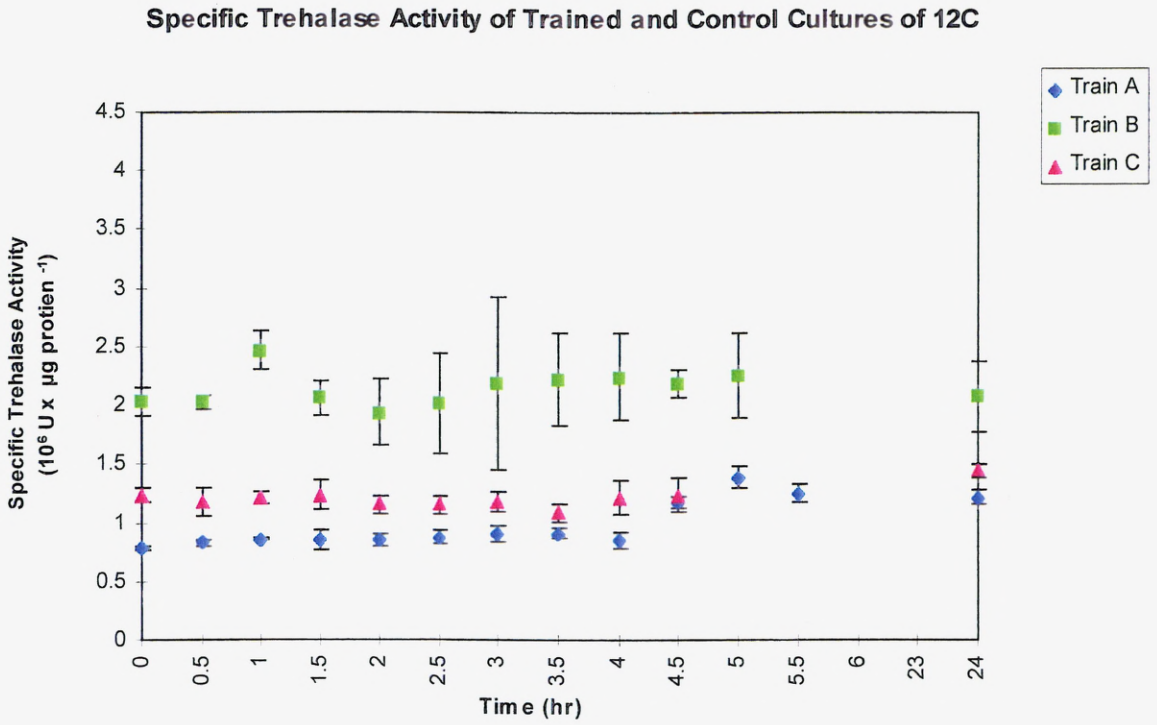


Figure 3.44. Specific trehalase activity of 12C Train A, B and C cultures which have undergone extreme heat treatment and recovery periods. Cultures were harvested every 30 min and specific trehalase activity was determined whereby 1 Unit of trehalase activity is defined as the amount of enzyme which gives rise to 1 nmol of glucose/ min/ µg protein. Train A: 0 hr represents cultures harvested after training at the sublethal temperature regime of 28 ^{0}C/ 24 hr; 0.5 - 2 hr period represents Train A cultures harvested during exposure to extreme heat treatment (40 ^{0}C/ 2 hr); the period between 2.5 - 24 hr represents cultures harvested during the recovery period (22 ^{0}C/ 24 hr). Train B: 0 hr represents cultures harvested after sublethal temperature regime of 25 ^{0}C/ 48 hr; 0.5 - 2 hr period represents Train B cultures harvested during exposure to extreme heat treatment (40 ^{0}C/ 2 hr); the period between 2.5 - 24 hr represents cultures harvested during the recovery period (22 ^{0}C/ 24 hr). Train C: 0 hr represents control cultures harvested after incubation at optimal conditions of 22 ^{0}C/ 24 hr; 0.5 - 2 hr period represents control cultures harvested during exposure to extreme heat treatment (40 ^{0}C/ 2 hr), the period between 2.5 - 24 hr represents cultures harvested during recovery period (22 ^{0}C/ 24 hr).

Exposing 12C cultures to sublethal temperature regime (Train A), appears to have no major effect on the level of trehalase activity throughout extreme and recovery periods. This is clearly demonstrated in Figure 3.44, with an almost constant level of activity at a value of approximately 0.9 x 10^6U/μg protein, which is similar to that found in Bb24 Train A counterparts (Figure 3.43). The only comparative difference to Bb24 Train A, was a slight increase in activity at 4.5 hr, which gradually decreased down to 1.22 x 10^6U/μg protein at 24 hr. The overall level of activity was less than that found in Train C, which is similar to findings for Bb24 Train A cultures (Figure 3.43). Exposing cultures to sublethal temperature of 30 ^{0}C for 48 hr (2.037 x 10^6U/μg protein at 0 hr), had the greatest effect on the level of trehalase with 1.6 to 2.5 fold increase compared to Train A and C counterparts respectively. As with 12C Train A and control cultures, there are no distinct changes in the level of activity where the values fluctuate between approximately 2.0 and 2.2 x 10^6U/μg protein, with exceptions at 1 hr and 2 hr periods of 2.471 and 1.938 x 10^6U/μg protein respectively. Train B cultures appear to demonstrate the highest level of activity compared to both Train A and C cultures, with nearly a 2.5 and 1.6 fold increase at 0 hr (Table 3.3.9), respectively. This ratio in the difference in levels continues throughout the time courses of Train A, B and C, which is clearly illustrated in Figure 3.44.

3.57 Correlation's Between the Levels of Trehalose and Trehalase Activity in Cultures Subjected to Sublethal (Trained), Extreme and Recovery Temperature Regimes.

To summarise, a number of correlation's can be demonstrated when comparing the changes in the levels of trehalose and neutral trehalase activity. The level of trehalase activity present during the time course for Bb24 Train B cultures starting at 0 hr period follows a pattern whereby there is reduced activity during extreme heat treatment (Figure 3.39), which corresponds to high levels of trehalose during the same period (Figure 3.43). Upon transfer to recovery conditions, increased levels of trehalase activity occur with an overall decrease in trehalose (Figures 3.39 and 3.43 respectively). This trend is apparent only in Bb24 Train B cultures, with no similar patterns occurring in any of the other respective sublethal training regimes for both Bb24 and 12C.

In control Bb24 cultures (Train C), trehalose levels are low (0 hr) which can be correlated to comparatively high levels of trehalase activity during the same period (Figure 3.43). The opposite of this trend occurs for Bb24 Train A cultures sampled at 0 hr, with a low level of trehalase activity, associated with an increase in trehalose levels above the control values (Figure 3.39 and Figure 3.43, respectively). No apparent inverse relationship can be demonstrated between the changing levels of trehalose and trehalase activity during extreme treatment and recovery regimes (0.5 - 3 hr and 3.5 - 24 hr, respectively) in Train A cultures. There is a noticeable decrease in trehalose levels from 4.5 - 24 hr, compared with no apparent increase in trehalase activity in the same period.

When comparing the levels of trehalose and respective hydrolysing enzyme in 12C Train, B and C cultures, a different trend to that found for Bb24 is observed. The low levels of trehalose (Figure 3.40) throughout the time course (0 - 24 hr) for Train B cultures corresponds

with sustained high levels of trehalase activity during the same period (Figure 3.44). In the case Train A cultures, sustained high levels of trehalose correspond to low levels of trehalase activity. Train C cultures follows a similar pattern to Train A, but to a lesser extent, although there is a decrease in trehalose levels at 24 hr, which corresponds with a slight increase of trehalase activity during the same period.

When monitoring the levels of both trehalose and trehalase in cultures of Bb24 and 12C, planning of the experiment took into consideration that measurements made, originated from a single culture. Therefore determining the method of sample preparation for both extraction protocols was crucial, as trehalose extraction was by far more stringent to that of the enzyme extraction (refer to Chapter 2, Material and Methods, Sections 2.6.2 and 2.6.4). To be able to separate the two methods, the starting material had to be divided and accurately measured. Freeze drying the cultures became the method of choice for ease of handling the starting material as it was difficult to divide snap frozen material which defrosts at 4 ^{0}C. The accuracy of measuring out approximately 5 mg of frozen material for trehalose analysis was also difficult due to the excess water content. If freeze drying the material had an effect on the levels of trehalose and trehalase activity present in the cultures, this would have possibly been illustrated by similar values occurring for all the cultures sampled. Therefore no real distinct changes between the various time courses for Train A, B and C cultures for both species would have been demonstrated.

CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

4.0 Changes in Protein and mRNA Expression.

It has been demonstrated in mammalian cells that the effects of elevated temperature result in the inhibition of macromolecular mechanisms, such as DNA, RNA and protein synthesis (reviewed in Roti Roti and Laszlo 1988). The response of Bb24 and 12C to elevated temperatures was investigated by analysis of the patterns of proteins synthesised shown in Figure 3.1 A, B, C and D. Overall protein synthesis was detected and measured by the incorporation of [^{35}S] methionine into TCA-precipitated material (Table 3.1).

Together, these observations indicated strongly that sustained protein synthesis during extreme heat treatment is dependent on pre-conditioning cultures to sublethal training regimes (Train A and B), for Bb24 cultures only (Figure 3.2). The sublethal training regimes used on 12C did not effectively induce thermotolerance as previously suggested by the growth rate studies of White et. al. (1995), as the relative protein synthesis during recovery was negligible. It was demonstrated during extreme treatment for both Bb24 and 12C control cultures respectively, that protein synthesis was negligible corresponding to very high levels of HIIPS (Table 3.2). After 24 hr in recovery a distinct difference in protein synthesis was illustrated between the two species as resumption of protein synthesis occurred for *S. himantioides* only (as illustrated by the reactivation of protein synthesis). This indicated that the extreme heat regime of 40 ^{0}C for 3 hr used in this study, did not effectively kill Bb24 however, it is clear that 40 ^{0}C/ 2hr did effectively inhibit cellular activities in *S. lacrymans* White et. al. (1995), as after 24 hrs in recovery, resumption of normal protein synthesis did not occur. The observations from these metabolic studies also substantiates the comparative difference of the two *Serpula* species observed by Seehann and Riebesell (1988), suggesting the overall thermotolerant nature of *S. himantioides* to that of *S. lacrymans*. One study carried out by Higgins and Lilly (1993) attempted to show the multiple responses associated with heat stress in the

basidiomycete *S. commune* by the analysis of hsp production, protein ubiquitination and changes in proteases. The conditions used to initiate heat stress were comparatively similar to those used in this study, with extreme temperature regime of 55 ^{0}C for 3 hr compared to 40 ^{0}C for 2 and 3 hrs (12C and Bb24 respectively).

The systematic band comparisons of the 1-D autoradiograms illustrated the complex pattern of synthesis, which occurred for both *Serpula* species. Global comparisons were made illustrating the similarities between Bb24 and 12C (Figure 3.6 A and B). The expression of proteins whose molecular weights are similar to those of members of the Hsp Families were highlighted for both Bb24 and 12C (Table 3.2.1), a distinct difference between the two species was the presence of high molecular weight proteins which fall within the Hsp 70 and 90 M_r range in Bb24 only. However it was interesting to note that both Bb24 and 12C produced high molecular weight proteins following mRNA extraction and *in vitro* translation (Figure 3.12). The largest group of proteins which were heat-induced and constitutively expressed in both Bb24 and 12C fell within the molecular weight range of that of the small Heat-Shock Proteins (sHSPs). There was a range of constitutively expressed bands corresponding in molecular weights to a number of small hsps is consistent with evidence suggesting a functional role for these proteins in normal cell metabolism and development (Rossi and Lindquist, 1989; Stahl et. al., 1992; Gernold et. al., 1993). Constitutively and tissue specific low level expression of small hsps have been detected in unstressed tissues (Glaser et.al., 1986; Bielka et. al., 1988; Hammerschlag et. al., 1989; Heikkila et. al., 1991; Marin et. al., 1993). Newly synthesised bands, which were heat-induced, corresponded in molecular weight to hsp27, were also identified in Bb24, but not 12C. These bands corresponding in molecular weight to 27.2 kD were expressed in only the sublethal (Train A and B), and subsequent extreme and recovery extracts (Table 3.1.3). The levels of expression for the 27.2 kD protein represented as %IOD for both trained extracts decreased during extreme heat-treatment, but increased during the recovery

phase (Table 3.1.5). This observation of increased levels of hsp27 expression after heat-shock has been demonstrated in mouse, Chinese hamster and rat glioma cells (Klemenz et. al., 1991; Landry et. al., 1991; Inaguma et. al., 1992). Studies have also shown that hsp27 accumulates with slower kinetics and are synthesised for longer after stress (Landry et. al., 1991; Klemenz et. al., 1993). In chick embryo cells, there was a notable difference in the concentration of hsp27 after heat-shock, in contrast to accumulation of hsp71 and hsp88 (Edington and Hightower, 1990).

Studies in mammalian cells have also suggested a role of hsp27 in thermotolerance. Stable heat-resistant variants were isolated after mutagenesis of CHO (Chrétien and Landry, 1988). Constitutive expression of the human hsp27 in Chinese hamster and mouse cell lines has also conferred protection from thermal killing (Landry et. al., 1989).

The overall recalcitrant nature of the mycelium resulted in inefficient protein extraction from both *Serpula* species, which clearly demonstrated by the quality of the 2-D autoradiographic profiles. The stringent procedure used in sample preparation for releasing intracellular material and the difficulties associated with protein solubility for 2-D analysis would have definitely played a major part in the inefficient separation and resultant resolution of proteins for Bb24 and 12C extracts. The sensitivity and power of 2-D SDS PAGE, was clearly illustrated for the analysis of total protein extracts from L-2226. Heat-induced proteins where detected only during sublethal (42 ^{0}C) and extreme temperatures (52 ^{0}C) could be clearly seen in relation to basal levels of proteins present throughout the temperature regimes imposed (Table 3.2.3).

4.1 Immunological Analysis for the Detection of Hsp60, 70 and Ubiquitinated Proteins.

Serpula species homologues for hsp60 and 70 were detected *in vivo* in cell extracts from Bb24 (Figure 3.15 A and 3.17 A) but not in those from 12C (Figure 3.15 B and 3.17 B) by polyclonal antiserum. There was no detectable presence in both species of a cross reactive product for the monoclonal antibody equivalents, possibly indicating a post-translational modification of the proteins in which the surface epitopes recognised by the MAb's are lost. This was further substantiated by the immunological analysis of the *in vitro* translational extracts whereby a signal was detected by both hsp60 and 70 MAb's for both *Serpula* species (Figures 3.21 and 3.22). The possible partial and complete loss of antigenic epitopes for hsp60 in Bb24 and 12C respectively can also be demonstrated by the comparative analysis of the intracellular and *in vitro* translation blots. A difference in signal size was detected by anti-GroEL serum for intracellular extracts for Bb24 (Figure 3.15 A), which ran at a lower molecular weight to the signal detected *in vitro* translation extracts by MAb 60 (Figure 3.21 A). In comparison with no signal seen for 12C for intracellular (Figure 3.15 B), but with a subsequent signal seen for *in vitro* translation extracts (Figure 3.21 B).

MAb's can be used for the identification of gene products in recombinant DNA experiments, where the gene product from such an experiment could be modified in such a way that the antigen has lost the corresponding epitope. An example illustrating this, is where a protein has been truncated or not correctly folded. Discontinuous or conformational epitopes, which are composed of different stretches of the polypeptide chains, should be particularly sensitive to incorrect refolding. Therefore, the epitopes of an antigen are not equally stable to denaturation and some might be difficult to renature after denaturation. This problem is not so serious when working with polyclonal antibodies, as their specificity is not as narrow with specificity to a broad range of epitopes.

The process where proteins labelled for degradation was investigated for both Bb24 and 12C. Protein degradation by the ubiquitin-proteasome degradative system in relation to the temperature regimes applied to Bb24 and 12C (Figure 3.23). It was demonstrated for 12C that a group of proteins ear-marked for protein degradation were present in extracts from cultures exposed to sublethal and extreme temperatures. To date the only other study using an anti-ubiquitin serum to monitor changes in protein degradation is by Higgins and Lilly (1993). Their results indicated changes in a ladder of low molecular weight ubiquitinated conjugates from heat-shock cultures of *S. commune*, with high molecular weight conjugates appearing to be less abundant.

To date the immunological detection of hsp 60 and 70 in similar basidiomycetes has not been undertaken and there is no corroborative evidence to which a feasible explanation as to why these two *Serpula* species behave in the manner illustrated.

4.2 Detection and Identification of Hsp70.

The detection of a 70 kD protein by immunoblot analysis on the 1D intracellular autoradiograms and resultant immunoblots for Bb24 extracts, but none for 12C for intracellular extracts. The analysis of mRNA expression by *in vitro* translation studies did however produce a 70 kD band for both Bb24 and 12C extracts, this was further substantiated by a positive signal detected for both species by MAb 70.

To confirm whether the identification of a 70 kD band by immunoblot detection in both intracellular and *in vitro* translation extracts, a molecular based approach using sequence specific primers was initiated identification using sequence specific primers derived from the *A. bisporus* hspA (hsp70) sequence. By RT-PCR and PCR based methods, cDNA and genomic DNA fragments respectively were produced which correlated in size to the control *A. bisporus* fragments (Figure 3.27).

The rationale behind the experiments undertaken, demonstrated the following: (i) the optimisation of the RT-PCR and PCR reaction procedure resulted in the highest level of efficiency possible; (ii) the PCR products derived from both RNA and DNA starting material could be easily identified due to the careful design of the primers which span an intron; (iii) the reaction products were cloned and sequenced establishing that the control mushroom PCR product was related to the previously partially sequenced *A. bisporus* hspA gene (Schaap et. al., 1996).

Although the identification of 12C and Bb24 amplimer products by the sequence-specific primers generated from *A. bisporus* hsp70 gene sequence was achieved, the subsequent cloning and sequencing of the reaction products only gave positive identification of the control to that of the *A. bisporus* hspA gene sequence (Accession no. X98508). The generated amplimer DNA sequences for the two *Serpula* species closely resembled the DNA from the pGEM vector used in this study.

Further cloning experiments will have to be undertaken to verify whether the cDNA and genomic DNA sequence amplified by the primers are in fact fragments derived from a *Serpula* species are fungal homologues of the hsp70 gene.

4.3 Immunological Detection of AMPK.

To date there is no work detailing the role of AMPK in stress signalling in basidiomycetes. Immunological studies illustrated a cross reactive product detected from Bb24 intracellular extracts exposed to sublethal (Train A and B) and extreme temperature regimes (Figure 3.37 A). An increase in AMPK levels resulting from stress was clearly demonstrated for the control (Train C) set. With a cross reactive product identified during extreme-heat treatment only, which corresponds to a growing body of evidence that this protein kinase is activated only during periods of cellular stress (Moore et. al., 1991; Corton et. al., 1994; Hardie et. al., 1994; Hawley et. al., 1995). The lack of

activity measured in the cellular extracts of both species may be explained by one or more of the following reasons: dephosphorylation of AMPK; extraction causes inhibition of phosphorylation; the kinase may require *de novo* phosphorylation *in vivo*; the cross reactive product identified immunologically may not have the same peptide substrate as the AMPK of yeast, plant and animal systems (Woods et. al., 1994; Mackintosh et. al., 1992 and Davies et. al., 1989). To rule out whether the enzyme present in Bb24 and 12C is in an inactive state, further work will have to be carried out to purify the enzyme followed by subsequent experiments to evaluate the effect of deactivation by protein phosphatase C and reactivation by a kinase kinase assay (Moore et. al., 1991).

4.4 Changes in Trehalose and Trehalase Levels During the Heat-Shock Response.

From the overall analysis of trehalose present during the various temperature regimes imposed on *S. lacrymans*, no correlation could be made for a transient increase in trehalose and induced thermotolerance in any of the training regimes used for 12C cultures. Although sustained high levels present in Train A cultures with slightly lower levels seen for control (Train C), throughout the time course, may be an indication that 12C is in a state of permanent stress (Figure 3.40). Comparing the overall levels of trehalose in *S. himantioides* (Figure 3.39) could support this by linking the observation of *S. himantioides* having a far more thermotolerant nature to *S. lacrymans*, therefore the levels of trehalose required to protect the cells during periods of stress are less.

Bb24 cultures pre-treated at sublethal temperature regime Train B, shows increased levels of trehalose during heat treatment followed by a decrease in recovery corresponding to an increase in trehalase activity during the same period (Figures 3.39 and 3.43). This resembles similar trends which have been observed in other eukaryotic studies (Killick and Wright, 1972; Attfield, 1987; Hottiger et. al., 1987; De Virgilio et. al., 1991; Neves and Francois, 1992), where acquisition of

thermotolerance and accumulation of trehalose are closely correlated during preconditioning and extreme heat stress and could be postulated to correlate to the occurrence of induced thermotolerance.

It has been demonstrated that in exponentially growing yeast, there are basal levels of trehalose and low trehalase activity (Thevelein, 1991). Upon exposure to sublethal temperatures (40 - 45 ^{0}C for 1 to 2 hr), yeast rapidly accumulate large amounts of trehalose (Attfield, 1987; Hottiger et. al., 1987b) which has been shown to correlate with increased thermotolerance (Hottiger et. al., 1987a). However it has also been reported that a short severe heat-shock of 3 mins at 50 - 54 ^{0}C causes an apparent activation of neutral trehalase in yeast ascospores, but this may be an artifact arising during sampling which consists of rapidly cooling the cells in ice-cold water (Thevelein, 1984c). Hottiger et. al. (1987b) demonstrating that the enzyme measurements of trehalose-6-phosphate synthase and trehalase taken from extracts prepared by disrupting yeast cells with glass beads at 4 ^{0}C produced a high yield of both enzymes during heat-shock, suggesting a high turn-over of trehalose. The above studies indicate that there may be artificial activation of trehalase during sample preparation.

The possible problems associated with sample procedures was investigated (De Virgilio et. al., 1991b) by permeabilisation of cells through rapid freezing in liquid nitrogen and their subsequent thawing in the presence of Triton X-100. It was demonstrated that in extracts prepared at 40 ^{0}C and in phosphatase-treated buffer at 0 ^{0}C (dephosphorylation of enzyme), trehalase activity increased to a peak of 3 times higher than in control cells after 60 min. De Virgilio et. al., (1991b) also demonstrated by Western blot analysis, with MAb's against neutral trehalase showed a similar increase in the amount of enzyme present. The use of this procedure illustrated the rapid activation of trehalase upon a shift in temperature of 40 to 27 ^{0}C, which correlated with rapid degradation of accumulated trehalose, followed by rapid inactivation of trehalase when the cells were placed back into 40 ^{0}C and subsequent accumulation of trehalose.

4.5 Thermotolerance, Protection and Chemical Chaperones.

Despite the strong evidence linking induction of hsps to acquired thermotolerance, studies giving evidence of thermotolerance without new hsp synthesis also exist, indicating that cells have more than one mechanism for achieving this protective state. Borrelli et. al. (1996), demonstrated that mitotic mammalian cells whose chromosomes are condensed and essentially shut down, can still acquire thermotolerance without the production of hsps. The chemical chaperone trehalose can reach intracellular levels of 0.5 mol/l and together with hsp104 contribute to yeast thermotolerance (Elliot et. al., 1996). Yeast mutants lacking an enzyme in the trehalose biosynthetic pathway have been shown to be 500 fold more sensitive to heat-shock (Elliot et. al, 1996). It has also been demonstrated that trehalose stabilises proteins against thermal denaturation both *in vivo* and ***in vitro*** (Hottiger et. al., 1994b; Singer and Lindquist, 1998a). Trehalose has also been implicated in preventing aggregation of proteins, which appeared to be stabilised in a partially folded, molten globule-like form (Singer and Lindquist, 1998b).

The two aspects of the heat-shock response, hsp induction and trehalose metabolism, have been widely studied and both have been suggested to contribute substantially to the increase in stress tolerance associated with heat shock. Exposure of cells and organisms to elevated temperatures triggers the synthesis of hsps (Laszlo, 1988; Nover, 1991). It seems that hsps are prime candidates for molecules that protect cells from extreme temperatures as hsp synthesis and induction of thermotolerance are closely correlated (McAlister and Finkelstein, 1980; Li and Werb, 1982; Plesofsky-Vig and Brambl, 1985; Lindquist and Craig, 1988; Nover, 1991). Constitutive expression of hsp70 in mammalian cells confers thermotolerance (Angelidis et. al., 1991) in transformed cell lines also provided a direct means of studying the effects of selected hsp70 expression on cell

survival after heat-shock. As with hsp70, there is a correlation between hsp27 levels and thermal resistance (Landry et. al., 1989).

Smith and Yaffe (1991), also demonstrated *S. cerevisiae* cells expressing a mutant HSF protein, *hsf1-m3* resulted in the blockage of hsp induction but did not affect the acquisition of thermotolerance. Other studies show that thermotolerance is partly independent of hsp synthesis (Barnes et. al., 1990; De Virgilio et. al., 1991a). Both yeast and *Tetrahymena* cells treated with cyclohexamide (inhibitor of protein synthesis) prior to exposure to elevated temperatures, developed thermotolerance (Hall, 1983; Hallberg et. al., 1985). Treatment of CHO cells during hyperthermia and heat treatment provided the same degree of protection against cell killing at 43 ^{0}C as glycerol and thermotolerance (Borrelli, et. al., 1991, 1996) implying that cyclohexamide stabilises cellular proteins against thermal denaturation and thermotolerance occurred without the preferential synthesis of hsps (Borrelli, et. al, 1996).

Contradictory to the above findings, studies have shown that in *S. cerevisiae*, long-term survival at extreme temperatures has been linked to elevated levels of the hsp104 (Sanchez and Lindquist, 1990; Sanchez et. al., 1992). This contradicts the previous findings by Smith and Yaffe (1991), of the heat-shock transcription factor mutation blocking hsp induction without blocking thermotolerance. Lindquist and Kim, (1996) demonstrated that this mutation did not block the induction of hsp104, as it could not be deleted in *hstf1-m3* cells as the expression of heat-shock factor requires suppression mediated by the yeast prion, which in turn depends upon hsp104. As mentioned earlier there is also possible co-operation between hsp104 and the disaccharide, trehalose (Elliot et. al., 1996). However, while both hsps and trehalose are likely to be major factors involved in stress tolerance, the relative contribution of each and their mechanisms of action are still unclear (De Virgilio et. al., 1990; Watson, 1990; Craig et. al., 1993; Piper, 1993, Hottiger et. al., 1994b).

As heat and other stress conditions cause cellular proteins to become partially unfolded, the ability of heat-shock proteins to protect cells against these adverse effects by utilising their 'house-keeping' functions as molecular chaperones is still of major importance.

4.6 The Quest to Find New Innovative Ways to Study Hsp Expression.

At present the availability of antibodies to hsps that cross react with proteins from a wide variety of species has permitted the use of Western blotting techniques and other immunological methods to quantitatively measure constitutively-expressed and stress-induced hsps in organisms and cell lines (Lai, et. al., 1984; Landry et. al., 1989; Beckman et. al., 1992; Bloch and Johnson, 1995; Lindquist and Kim, 1996). This approach for the quantitative analysis of important hsps are relatively straight forward and biologically relevant with respect to direct measurement of epitopes which can be inferred to be an adaptive phenotype of hsps, with regards to the abundance of these proteins in relation to their possible protective roles in multi-stress responses. There are drawbacks to this method of analysis especially when considering the underlying molecular events associated with these responses which must be taken into consideration when tackling question of specific changes in hsp expression. The following points highlight the possible limitations linked to this form of analysis. (i), the hsp under investigation may not be detectable at a level immediately following exposure to a stress, given the time lag between transcription and translation of a particular gene. (ii), the stability of hsps on the whole are regarded as quite high, which may result in their continued presence even after the removal of the stressor.

The alternative to examining changes in protein expression, is the examination in changes in specific mRNA expression resulting from stress. Such an approach is based on the assumption that changes in gene expression, resulting in subsequent changes on protein abundance, typically involves changes in either the rate of synthesis or stability of mRNA levels. If these changes due to

stress affect the regulation of hsp genes at the transcriptional level, the first measurable change that occurs is at the mRNA level. Therefore, measurements at the mRNA level might provide a valuable means of detecting short-term responses to stress.

Due to the high degree of evolutionary conservation of stress proteins, the potential exists for the development of sequence specific nucleic acid probes from particular species that could be used to monitor stress-related changes in mRNA abundance. The application of PCR for the generation of these probes, provided that the primer sequences can be identified that specifically amplify sequences of interest from a wide variety of organisms. PCR is a powerful tool for the qualitative and quantitative monitoring of molecular changes within the cell. Considerable attention to changes in levels of gene expression in response to various stresses have been analysed involving primarily hybridisation assays to measure RNA abundance (Krone and Heikkila, 1988, Kusukawa and Yura, 1988; Werner-Washburne et. al., 1989, Borrelli et. al., 1996). The recent utilisation of RT-PCR based techniques due to its sensitivity and reproducibility is becoming increasingly used for analysis of RNA abundance with particular emphasis on specific hsp mRNAs (Rush et. al.,1990; Ohan and Heikkila, 1995; Zhao et. al., 1994, 1995; Gross and Watson, 1998). Oligo (dT), random (hexamer-) primers and the PCR antisense primer can be used as primers for the reverse transcription reaction. Oligo(dT) primers are often inefficient for transcripts with long 3' untranslated regions, as the efficiency of reverse transcription decrease as distance from the primer increases. Both oligo (dT) and random hexamer primers are not sequence-specific and therefore, reverse transcription from total cellular RNA may often be inefficient. An alternative lies in the use of sequence-specific primers. The design of the primers generated from specific gene sequence can help to eliminate these associated problems to detect only the hsp sequence in question.

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APPENDIX

APPENDIX A

A1 Antibody Specificity.

The specificity of the polyclonal and monoclonal antibodies used in the study were as follows:-

(i) Anti-Hsc/Hsp 70 mouse monoclonal antibody specific for the constitutive and inducible forms of Hsp 70 (Hsc and Hsp 70) that will recognise and cross react with family members from evolutionary divergent organisms of human, rabbit, mouse, dog, chicken, Drosophila and *S. cerevisiae* cell lines.

(ii) Anti-60kD hsp mouse monoclonal antibody specific for Hsp 60 will identify from primates, mouse, rat, hamster, rabbit, pig, *Borellia*, *E. coli*, *Streptococcus pyogenes*, *Yersinia enterolitica*, *Salmonella typhimurium*, *Treponema hyodysenteriae*, *T. innocense*, *Trichinella spiralis*, yeast extracts and spinach chloroplasts.

(iii) The immunogen used to anti-ubiquitin rabbit polyclonal antibody was ubiquitin purified from bovine red blood cells conjugated to KLH, the antibody has been shown to immunoprecipitate BSA-conjugated ubiquitin.

A2 Suppliers of Equipment and Chemicals

Amersham International Plc., Amersham Place, Little Chalfont, Buckinghamshire, UK, HP7 9NA.

Amresco, Solon, Ohio, USA.

BDH, Poole, Dorset, UK.

Bio-Rad Laboratories Ltd, Hemel Hempstead, Hertfordshire, UK, HP2 7TD.

Birchover Instruments Ltd, Bridge Road, Letchworth, Hertfordshire, UK, SG6 4ET.

Coulter Electronics Ltd., Northwell Drive, Luton, Beds., UK, LU3 3KH.

Fisons, FSA Laboratory Suppliers, Loughborough, UK.

Fluka Chemie A. G., CH-9470 Buchs, Switzerland.

Genetics Computer Group, 515 Science Drive, Madison, Wisconsin, 53711 U.S.A.

Genosys Biotechnologies (Europe) Ltd., London road, Pampisford, Cambridge, CB2 4EF England.

GIBCO/BRL, 3175 Staley Road, Grand Island, NY 14702.

International Equipment Company, Division of Damon, Dunstable, Bedfordshire, England.

Millipore (UK) Ltd, Watford, Hertfordshire, UK, WD1 8YW.

Non Linear dynamics Ltd., The Technopole, Trafalgar Street, Newcastle upon Tyne, England, NE1 2LA.

Oxoid, UNIPATH, Basingstoke, UK.

Pharmacia-LKB Biotechnology, Milton Keynes, Buckinghamshire, UK, MK9 3HP.

Pierce, Rockford, Illinois, USA.

Promega UK Ltd, Delta House, Chilworth Research Centre, Southampton, SO16 7NS.

Leica Cambridge Ltd., Clifton Rd., Cambridge, CB1 3QH, England.

SAPU, Law Hospital Carluke, Lanarkshire, Scotland, ML8 5ES.

Sartorius Ltd, GB-Belmont, Surrey, UK, ML8 5ES.

Schleicher and Schuell, 10 Optical Avenue, Keene, NH 03431.

Sigma Chemical Company Ltd, Poole, Dorset, UK, BH17 7TG.

Stress Gen Biotechnologies Corp., The Raylor Centre, James Street, York, North Yorkshire, UK, YO1 3DW.

ThermoMetric Ltd., 10 Dalby Court, Gadbrook Business Park, Northwich, Cheshire CW9 7TN.